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Pathogenesis of Salmonellosis: *Salmonella* Exotoxins

Annual Progress Report

(9/1/79-8/31/80)

Johnny W. Peterson, Ph.D.

March 8, 1982

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University of Texas Medical Branch
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This annual progress report compiles research progress accomplished during the third year of research contract DAM D17 77 C 7054 covering the period 9/1/79-8/31/80. None of the progress reported here has been reported in prior periods. As indicated in the table of contents, a substantial portion of this progress is contained in two new manuscripts. The first manuscript describes two new in vitro assays that were developed to facilitate future studies of the characteristics

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SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered) of Salmonella toxin and its involvement in the pathogenesis of salmonellosis. One of the rapid, in vitro assays measures biological activity in terms of Salmonella toxin's capacity to stimulate adenylate cyclase in Chinese hamster ovary cells. The assay is a modification of the assay developed for cholera toxin by Nozawa et al. (18), and measures the enhancement of CHO cell adherence in cultured monolayers. Adherence of CHO cells for each other in a growing monolayer increases as intracellular cyclic AMP increases. The mechanism of adherence probably relates to enhanced synthesis of fibronectin, and adherence is a cyclic AMP mediated function (13). Technical performance of the assay is rapid, reproducible, and free of subjective assessments that complicate the earlier CHO cell assay of Guerrant (10). A coulter cell counter is employed to determine the number of cells "floating" above a CHO cell monolayer in plastic multiwell dishes. The number of floating cells is inversely proportional to the cyclic AMP content of the CHO cells. In each assay, a series of CHO cell wells are included which receive 50 1 dilutions of purified cholera toxin. From the floating cell responses of the cells in these wells, a dose response curve is plotted and responses of Salmonella toxin treated cells are expressed as cholera toxin equivalents in ng/ml.

The second rapid assay for Salmonella toxin described in the first manuscript is the enzyme-linked immunosorbent assay (ELISA). We strived to develop this assay to exploit the antigenic similarity between cholera toxin and Salmonella toxin, which was presented in an earlier project period. Like the CHO floating cell assay, the ELISA assay has proven to be very sensitive, which facilitates quantitation of the low concentrations of the Salmonella toxin. Two ELISA methods are presently used to measure Salmonella toxin-indirect and double antibody sandwich methods. The indirect method is described in detail in the first manuscript and employs monospecific cholera antitoxin, specifically purified from hyperimmune rabbit serum with a purified cholera toxin-agarose immunoadsorbent. The indirect ELISA measures the amount of Salmonella toxin antigen adsorbing to the wells of polystyrene microtiter plates. The Salmonella toxin antigen is then reacted with the purified cholera antitoxin solution. After washing, alkaline phosphatase-labeled goat antiserum to rabbit globulin is added. After extraneous proteins are washed away, the substrate p-nitrophenyl phosphate is added and the yellow reaction product (p-nitrophenol) is measured visually and spectrophotometrically at 400 nm. The intensity of reaction product formation is proportional to the toxin concentration. A series of purified cholera toxin dilutions are included in each assay, and all estimates of Salmonella toxin antigen are expressed as cholera toxin equivalents in ng/ml. In this manner, direct comparisons of Salmonella toxin concentrations can be made based on antigenic determinations (ELISA) and biological activity (CHO cell assay). A double antibody sandwich method has also been utilized in which burro antiserum to cholera toxin, diluted 1:10,000, is first coated onto the microtiter plates. With addition of this step, binding of cholera toxin and Salmonella toxin to the plates is a specific reaction. After that step, the assay is identical to the indirect assay just described. The latter assay has the advantage that it minimizes competition between the toxin and extraneous proteins for binding sites on the plastic plates. Furthermore, it is about 10 fold more sensitive.

The second manuscript describes our efforts to use the ELISA and CHO cell assay to study the effect of several cultural parameters on the synthesis and release of this heat labile, choleragen-like toxin from growing Salmonella cells. The study describes the production and release of Salmonella toxin in a classic growth curve experiment with and without the addition of mitomycin C (MTC). We had reported earlier that MTC increased the concentration of CHO cell toxin in crude culture filtrates of Salmonella species (12,16), but the mechanism was unclear. The second manuscript included in this report presents data to correlate the appearance of toxin in MTC culture filtrates with the simultaneous appearance of bacteriophage. These data support the observations of Gemski et al. (5) which indicated phage induction as the mechanism of toxin release in Escherichia coli cultures exposed to MTC. A survey of several Salmonella strains for toxin production was also included to help us select a strain for use in large scale production and purification of the toxin. We are continuing to search for other Salmonella isolates producing more toxin, and have included only the responses of representative isolates. It should be noted that the concentration of Salmonella toxin is measured in ng/ml, unlike cholera toxin which is elaborated in concentrations as high as 10-20 g/ml (2). It is for this reason, that we have delayed purification studies until we have exhausted all possible cultural parameters that would enhance the yields of the Salmonella toxin.

The remainder of the annual progress report summarizes significant and successful observations made during this period that have not yet been organized into publication format. The first section reflects a study of toxin production in a chemically defined medium and the stimulatory effects of vitamins, amino acids, and carbon sources on toxin synthesis. This study may aid in the development of a simple, inexpensive culture medium for toxin production that will not complicate purification work. We have successfully used such a medium for large scale cholera toxin production for several years (11,14).

Listed as a Specific Aim of last year's research proposal, we have strived to develop rapid in vitro tests to measure Salmonella toxin in culture filtrates and in cell sonicates. Our purpose was to develop the methodology for studying toxin characteristics and cultural parameters as well as facilitating detection of toxin during future purification procedures. We are very pleased with this aspect of our studies, and while this work progressed, in vivo studies have also been developing. Included in this progress report is a section relating to live Salmonella challenge of rabbit intestinal loops and subsequent measurement of the increased level of cyclic AMP in the mucosal tissue. Concentrated preparations of Salmonella filtrates as high as 30x fail to elicit a fluid accumulation response. Because of this, we have not tried to measure cyclic AMP responses in these loops. However, immediately following this section of the report, some interesting observations are included that demonstrate the elevation of cyclic AMP levels in cultured Henle intestinal epithelial cells exposed to filter sterilized Salmonella culture fluid. Similarly, we have demonstrated direct stimulation of adenylate cyclase in pigeon erythrocyte lysates and subsequent increase in cyclic AMP by filter sterilized, Salmonella culture fluid. We believe that a substantial portion, if not all of the cyclic AMP arising in rabbit intestinal loop tissue (7), may be due to the Salmonella toxin and its effects on epithelial cell adenylate cyclase. The data acquired thus far in cultured cells would tend to support this hypothesis, and argue against the role of the tissue inflammatory response in generating this cyclic AMP as proposed by Giannella et al. (7). A portion of the new proposal will address this aspect of the work.

A couple of agar plate assays have been developed to aid future genetic studies of Salmonella toxin. Both techniques involve specifically purified cholera antitoxin. One utilizes the concept of autoradiography while the other uses sheep erythrocytes and guinea pig complement. We are presently evaluating the performance of these procedures in detecting toxin from V. cholerae, E. coli, and Salmonella species.

Finally, we have noted another new factor produced by Salmonella, while studying the effects of concentrated culture filtrates on CHO cells and Vero cells. Some crude preparations appear to damage tissue culture cell monolayers in a fashion analogous to a cytotoxin. The factor produces reproducible effects and can be destroyed by boiling the filtrates. Preliminary purification data for the heat labile, cholera toxin-like toxin revealed that the cytotoxic factor could be separated out. This factor could be of some significance in that it might assist the Salmonella in penetration of the epithelium or be responsible for disruption of the brush border at the site of penetration described by Takeuchi (19). Future research may reveal the potential importance of this factor and its relationship, if any, to the cholera toxin-like toxin. Available progress suggests that the genetic information coding for the cholera toxin-like toxin in Salmonella is not located in any temperate bacteriophage genome. Rather, the role of phage, when activated, appears to be the lysis of cells and release of toxin into the medium.

Progress Report
DAM D17 77 C 7054

Annual Progress Report

Contract Number:	DAM D17 77 C 7054
Title:	Pathogenesis of Salmonellosis: Salmonella Exotoxins
Author:	Johnny W. Peterson, Ph.D.
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Salmonella Toxin: A Study of Some of the Biological, Biochemical, and Antigenic Characteristics

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Running Head: *Salmonella* toxin

Acknowledgements

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ABSTRACT

The ELISA and CHO floating cell assays for cholera toxin have proven sensitive and reliable in determining antigenic and biological characteristics of Salmonella toxin, respectively. The addition of mitomycin C (MTC) to the culture medium three hours after inoculation resulted in increased amounts of Salmonella toxin in culture filtrates but had the reverse effect on cell sonicates. These data suggest that increased amounts of Salmonella toxin in culture filtrates caused by MTC were due to cell lysis resulting in the release of intracellular toxin rather than an increase in the synthesis of Salmonella toxin. The biological activity of the toxin was heat labile at temperatures of 65°C and above. The antigenic structure appeared to remain intact after exposure to temperatures as high as 100°C but was altered somewhat when subjected to autoclaving. The toxin had an isoelectric point in the pH range of 4.3 to 4.8 and an estimated molecular weight which appeared to be greater than 110,000 daltons. With the exception of the range for its isoelectric point, molecular weight, and its low concentration in filtrates and sonicates, Salmonella toxin appeared to be very similar in biological, biochemical, and antigenic characteristics to cholera toxin which has an isoelectric point near pH 7. The antigenic and biological assays described here will provide an effective basis for extending our study of Salmonella toxin.

Introduction

Salmonella Toxin: A Study of Some of the Biological, Biochemical, and Antigenic Characteristics

Several genera of enteric bacterial pathogens associated with diarrheal disease release enterotoxins responsible for the loss of fluid and electrolytes from the intestinal mucosa. The best characterized of these enterotoxins are the heat-labile toxins of *Vibrio cholerae* and *Escherichia coli* (1,3,5,6,12,13). The mechanism of action of these antigenically related toxins involved stimulation of adenylate cyclase and the elevation of intracellular levels of cyclic adenosine monophosphate (cAMP). Guerrant and coworkers (8) have shown that *E. coli* and *V. cholerae* enterotoxins can elevate intracellular concentrations of cAMP in Chinese Hamster Ovary (CHO) cells; these changes in cyclic nucleotide metabolism were correlated with morphological alterations in the cells, thus forming the basis for a simple assay to detect these enterotoxins.

Extensive investigations of *V. cholerae* and *E. coli* enterotoxins has led to the concept that other enteric bacteria may also elaborate adenylate cyclase stimulating toxins. Koupal and Deibel (11) reported in 1975 an enterotoxic factor from *Salmonella* species that caused fluid accumulation in the suckling mouse. Independently, Sandefur and Peterson (17) demonstrated a delayed-acting skin permeability factor, now referred to as *Salmonella* toxin; this toxin was heat-labile and altered vascular permeability in the skin of adult rabbits in a manner indistinguishable from that of *V. cholerae* and *E. coli* enterotoxins. This toxin was recently shown to possess enterotoxic activity in adult rabbits (Peterson, J.W. et al. Manuscript submitted for publication).

Sandefur and Peterson (18) demonstrated the neutralization of the vascular permeability activity of Salmonella toxin with monospecific cholera antitoxin. In addition, Salmonella toxin was shown to be responsible for the elongation of CHO cells in a manner identical to cholera toxin.

The purpose of this investigation was to initiate a study of the synthesis and release of Salmonella toxin as well as its biochemical characteristics utilizing two very sensitive and reliable assays for monitoring the biological activity and antigenic presence of the toxin. The biological activity of Salmonella toxin was detected with the Chinese hamster ovary floating cell assay (CHO floating cell assay), which was a modification of the CHO floating cell assay for cholera toxin devised by Nozawa and coworkers (14). The antigenic presence of the toxin was monitored by the enzyme-linked immunosorbent assay (ELISA), which was a slight modification of the method described by Voller *et al.* (19).

MATERIALS AND METHODS

Organisms. Two strains of *Salmonella typhimurium*, SL1027 and SR11, were supplied by Drs. Samuel Formal, Walter Reed Army Medical Center and L. Joe Berry, University of Texas at Austin, respectively. Four clinical isolates of *Salmonella enterididis*, which included 9630 serotype Newport, 9186 serotype Newport, 10016 serotype Javiana, and 8994 serotype Braenderup, were supplied by the Houston Health

Department.

Preparation of cultures. Flasks containing 50 ml of Casamino acid yeast extract (CYE) medium (Molina, N.C. and J.W. Peterson, Manuscript in press, IAI 1148) were each inoculated in duplicate with one of the following strains of *Salmonella*: 9186, 10016, 9884, SL1027, and SR11 from slants of CYE agar. *Salmonella* strain 9630 was inoculated into a 250 ml flask containing 100 ml of Trypticase Soy broth (TSB). Flasks containing each medium were incubated with mild shaking (100 r.p.m.) at 37°C for 3 hrs before the addition of mitomycin C (MTC) to a final concentration of 0.5 µg/ml (Molina, N.C. and J.W. Peterson, Manuscript in press, IAI 1148). The flasks were incubated for an additional 21 hours with mild shaking at 37°C.

Preparation of filtrates. After the 24 hour incubation period, the cultures were centrifuged at 12,000 Xg for 10 minutes and the supernatants were filtered through 0.20-µm sterile Nalgene filter units. The filtrates were placed into sterile plastic tubes for storage at 4°C. One filtrate of *Salmonella* strain 9630, which was grown in the presence of MTC (9630 MTC), was concentrated fifteen fold by dialysis in 20M Carbowax (Union Carbide) followed by dialysis of the concentrate in phosphate diluent (P.D.) buffer pH 7.0, which has the following components per liter: 8.0 grams of NaCl, 0.2 grams of KCl, 1.15 grams of Na_2HPO_4 and 0.2 grams of KH_2PO_4.

Sonication. *Salmonella* cells were removed from the filtrates, washed one time with 40 ml of P.D. buffer and resuspended in 10 ml of P.D. buffer prior to sonication. Each preparation was submerged in an ice bath and sonicated with a Branson sonifier equipped with a microtip at 70 watts for 5 minutes. The sonicates were centrifuged at 15,000 Xg for 15 minutes and sterilized by filtration in 0.2 μm Nalgene filter units. The resulting supernatants were assayed for both antigenic and biological activities utilizing the ELISA and CHO floating cell assay, respectively.

Enzyme-linked immunosorbent assay (ELISA). On the basis of the observation that *Salmonella* toxin shares antigenic determinants with cholera toxin, the ELISA was employed to detect *Salmonella* toxin utilizing a specifically purified antibody to cholera toxin. The method of Voller *et al*. (19) with slight modifications was utilized in this study to detect *Salmonella* toxin antigen. Crude filtrates containing *Salmonella* toxin were mixed with an equal volume of carbonate buffer pH 9.6 (0.015 M Na_2CO_3 and 0.035 M $NaHCO_3$) and 200 μl of the mixture was allowed to adsorb to the wells of a polystyrene microtiter plate (Cooke) during an overnight incubation in a humid chamber at room temperature. A washing procedure involved removing the contents of the wells, refilling the wells with PBS-Tween buffer pH 7.4, and letting the wells stand for 3 minutes. This washing procedure was repeated for a total of three times. A volume of 200 ul of cholera antitoxin (1.5 mg/ml), produced in rabbits and specifically purified with a cholera toxin immunoadsorbent (9,15) was added in a 1:50 dilution with PBS-0.05% Tween "20" buffer to the wells and allowed to react with the adsorbed toxin for two hours. The wells were then washed as described previously. A 200 ul volume of goat antirabbit immunoglobulin conjugated with alkaline phosphatase (Miles Laboratories) was applied in a 1:1,000 dilution of PBS-Tween buffer to the wells and allowed to react with the cholera antitoxin for two hours. Finally, after washing the wells, 200 µl of the colorless substrate p-nitrophenyl phosphate (1 mg/ml of carbonate buffer, pH 9.6) was added and the reaction allowed to progress at 25°C for 30 minutes. The reaction was stopped by the addition of 50 µl of 3M NaOH. The p-nitrophenol released was measured spectrophotometrically at 400 n.m. Dilutions of purified cholera toxin with carbonate buffer were utilized as standards in the ELISA and antigen contained in crude Salmonella filtrates, sonicates, or fractions was determined and plotted against the cholera toxin standards. The Salmonella toxin values were expressed as cholera toxin equivalent units and reflect total antigen.

Chinese Hamster Ovary (CHO) floating cell assay. A modification of the CHO floating cell assay devised by Nozawa and coworkers (14) was used in this study. Each well of a plastic Linbro multidish was inoculated with 2.0×10^5 CHO cells per cm^2. The cells formed a monolayer after 24 hours incubation at $37^{\circ}C$ with 5% CO_2. After rinsing the monolayer with P.D. buffer, fresh medium with or without toxin was added and the cells were incubated for an additional 18 hours. During this time, proliferating CHO cells, which were not subjected to the toxin, floated off the monolayer into the medium whereas those wells inoculated with the toxin had more cells attached to the monolayer and fewer floating cells. The medium (0.5 ml) containing the floating cells was removed and the monolayer was washed one time with 1 ml of P.D. buffer. The medium and the wash were added to accuvettes containing 8.5 ml of P.D. prior to enumeration of floating cells with a Coulter counter (Figure 1).

Heat Study. Crude filtrate from Salmonella strain 9630, which was treated with MTC was tested for the heat stability of both its antigenic as well as its biological activities. Sealed tubes with 1 ml aliquots of filtrate were incubated for 1 hour at one of the following temperatures: 4°C, 37°C, 50°C, and 65°C. A sealed tube containing a 1 ml portion of Salmonella filtrate was also incubated at 100°C for 10 minutes and another 1 ml portion of Salmonella filtrate was subjected to autoclaving (121°C for 15 minutes). In addition, sonicates and filtrates of the following clinical isolates of S. enteritidis and S. typhimurium were heated by autoclaving for 15 minutes: 8994, 10016, 9186, SR11 and SL1027. The autoclaved filtrates and sonicates were tested for residual biological activity by the CHO floating cell assay and antigenic stability by the ELISA.

Column Chromatography. Partial purification of crude filtrate from Salmonella strain 9630, which was treated with MTC, was accomplished by gel filtration through a calibrated column (1.5 x 100 cm) of Sephadex G-150 (Pharmacia) equilibrated with P.D. buffer at pH 7.0. Two ml fractions were collected and the optical density was monitored at 280 nm. The column was calibrated with each of the following protein standards: 5 mg of ribonuclease A (13,700 daltons), 5 mg of chymotrypsinogen (25,000 daltons), 5 mg of ovalbumin (45,000 daltons), 5 mg of bovine serum albumin (67,000 daltons), 5 mg of Aldolase (158,000 daltons), 5 mg of ferritin (440,000 daltons), and 1 mg of blue dextran (2,000,000 daltons). To estimate the molecular weight of the Salmonella toxin, the elution volumes of the toxin fractions containing maximum biological activity, as determined by the CHO floating cell assay, were compared with the optical density peaks of the protein standards with known molecular weight.

Preparative isoelectric focusing. Approximately 1 mg of dialyzed filtrate from Salmonella strain 9630 MTC was applied to a preparative isoelectric focusing flatbed containing Sephadex G-75 superfine. The filtrate was subjected to 16 hours of isoelectric focusing with a constant power of 8W at $4^{\circ}C$ employing an ampholine range of pH 3.5 to 5.0. The flatbed was divided into 30 fractions in which the pH was measured with a surface electrode and dialyzed against P.D. buffer to remove the ampholines. The antigenic as well as the biological activity of the resulting fractions were monitored by the ELISA and CHO floating cells assays, respectively.

RESULTS

A standard curve was devised utilizing various concentrations of purified cholera toxin in the CHO floating cell assay as indicated in Figure 2. This assay was capable of detecting as little a 0.01 ng of cholera toxin. As the concentration of cholera toxin was increased, a smaller number of CHO floating cells was present in the culture medium; however, a larger number of floating cells was obtained when the monolayer was not exposed to cholera toxin. Photomicrographs of fixed and stained cells of the monolayer after removal of the floating cells are illustrated in Figure 3. The photomicrograph on the left displays normal CHO cells whereas the photomicrograph on the right indicates cells that were treated with 0.11 ng of cholera toxin. The toxin treated cells appeared elongated compared to the normal CHO cells.

A standard curve employing various concentrations of purified cholera toxin was utilized in the ELISA as shown in Figure 4. As illustrated in this figure, the ELISA was capable of detecting quantities as small as 0.37 ng of cholera toxin. The amount of p-nitrophenol released was directly proportional to the amount of antigen present over a two log range. The same standard curve was obtained when two different concentrations of alkaline phosphatase-labeled goat anti-rabbit globulin was employed in the ELISA. An experiment was designed to determine the specificity of the ELISA and the results are shown in Table 1. Various antigens were assayed with and without specifically purified cholera antitoxin as the second step in the ELISA. Positive readings (O.D. 400n.m. $\geq$ 0.1) were observed only when cholera antitoxin was employed in the ELISA; however, all readings from the ELISA when no cholera antitoxin was present were negative.

Molina and Peterson (manuscript in press, IAI # 1148) have demonstrated that the addition of 0.5 μg/ml of mitomycin C (MTC) in the culture medium of Salmonella strains after a 3 hour incubation period, increased the amount of Salmonella toxin in filtrates as demonstrated by the elongation of CHO cells. A similar experiment as shown in Figure 5 was conducted utilizing the ELISA to detect Salmonella toxin antigen in culture filtrates and cell sonicates. The Salmonella toxin concentration was expressed as cholera toxin equivalent units (ng/ml) since the ELISA values obtained for the Salmonella preparations were plotted against the cholera toxin standard curve. The addition of MTC enhanced the amount of Salmonella toxin present in the filtrates. Autoclaving destroyed some of the antigenic integrity of the toxin found in the filtrates as detected by the ELISA. When the sonicates were assayed by the ELISA for Salmonella toxin, no greater amount of toxin was present in the control group than in the group grown in the presence of MTC. The antigenic structure of the Salmonella toxin present in the sonicates was also affected somewhat by autoclaving as determined by the ELISA.

The same filtrates and sonicates of Salmonella strains grown in the presence or absence of mitomycin C and tested by the ELISA procedure were also tested in the CHO floating cell assay (Figure 6). The Salmonella toxin values were also expressed as cholera toxin equivalent units (ng/ml). Although the values differed for each strain tested, the filtrates of all strains grown in the presence of MTC demonstrated larger amounts of toxin present than in the control preparation. Autoclaving destroyed most of the biological activity of the toxin found in the filtrates. The data further indicated that in all strains more toxin activity was detected in the control group of cell sonicates than in cell sonicates that had been exposed to MTC during growth. The biological activity of the toxin found in the sonicates was almost completely destroyed when subjected to autoclaving. A protein determination of the filtrates and sonicates of five strains of Salmonella grown with and without MTC revealed the data in Table 2. Salmonella strains grown in the presence of MTC had a total protein content in the range of 4 to 40 mg in filtrates whereas most of the protein in Salmonella strains grown in medium without MTC was found in sonicates of the cells. This suggests that a large amount of non-toxin proteins are released into the medium in the presence of MTC.

To determine if MTC caused an increase in synthesis of Salmonella toxin, further analysis of data from Figures 5 and 6 was performed and summarized in Table 3. The average amount of biologic activity lost from the cells (sonicates) was not significantly different from that appearing in filtrates ($p>0.5$). The estimate for Salmonella toxin antigen gained in filtrates, as determined by the ELISA, appeared to significantly exceed that lost from the whole cells ($p=0.05$). In addition, in response to MTC there was no significant difference between the amount of Salmonella toxin antigen (ELISA) gained in the filtrates and the biologic activity (CHO cell assay) gained in the filtrates or lost in the sonicates ($p>0.5$).

A reduction by approximately 50% of the biological effect on CHO cells was observed when MTC filtrates of Salmonella strain 9630 were heated to a temperature of 65°C or above as illustrated in Figure 7. The antigenic structure of the Salmonella toxin as recognized by the ELISA remained intact when subjected to heating at this same temperature.

Filtrates and sonicates of five other strains of Salmonella (8994, SR11, SL1027, 1C016, and 9186) were tested for Salmonella toxin heat lability and found to lose most of their biological activity (95%) when subjected to autoclaving for 15 minutes as determined by the CHO floating cell assay. Autoclaving altered the antigenic structure an average of 35% for Salmonella toxin from the filtrates and an average of 64% for Salmonella toxin from the sonicates as detected by the ELISA (Table 4).

The application of approximately 1 mg of the filtrate of Salmonella strain 9630 MTC to a preparative isoelectric focusing flat-bed with an ampholine range of pH 3.5 to 5.0 resulted in the data presented in Figure 8. The Salmonella toxin antigen as determined by the ELISA was located in the range of pH 4.3 to 4.8. The biological activity of the Salmonella toxin antigen as measured by the CHO floating cell assay was located in the same region despite the absence of a significant protein peak.

Partial purification of the Salmonella toxin from the culture medium and other products of Salmonella was achieved by the application of 3 ml of concentrated (15X), cell-free filtrate from Salmonella enteriditis strain 9630 to a Sephadex G-150 column. Figure 9 illustrates the elution pattern of the concentrate. When fractions were tested by both the ELISA and CHO floating cell assay for antigenic and biological activity respectively, we found antigenic and biological activity associated with the first peak.

DISCUSSION

The addition of MTC enhanced the amount of Salmonella toxin present in the filtrates as determined by the ELISA and CHO floating cell assay. In addition, the protein content increased in the Salmonella filtrates due to the addition of MTC. There are at least two current explanations for the MTC phenomenon. Isaacson and Moon (10) postulated that MTC induced heat-labile toxin synthesis in enterotoxigenic strains of

Escherichia coli by mitomycin C was due to plasmid gene derepression. On the other hand, Gemski et al. (7) have reported the cellular release of heat-labile enterotoxin of Escherichia coli by bacteriophage induction. The data from our studies tend to support the latter mechanism, since MTC did not appear to enhance toxin synthesis. A decrease in turbidity of broth cultures of Salmonella strains grown in the presence of MTC was detected by visual observation. Studies are presently being conducted to determine whether the cell lysis was due to an MTC induction of bacteriophage.

When the Salmonella cells, which produced the toxin found in the filtrates, were sonicated, no greater amount of toxin was detected in the control group than in the group grown in the presence of MTC as determined by the ELISA. The biological activity of Salmonella toxin found in the control group sonicates for each strain of Salmonella was higher than that of the group grown in the presence of MTC as demonstrated by the CHO floating cell assay. Data from a protein determination indicated more protein in the sonicates of the control group than in the group grown in the presence of MTC. There was no significant difference in the average biologic activity lost from the cells (sonicates) than that found in the filtrates. In addition, there was no significant differences between the amount of Salmonella toxin antigen gained in filtrates compared to the biologic activity gained in the filtrates or lost in the sonicates (Table 3). The reason for the difference in levels of Salmonella toxin antigen found in filtrates and sonicates, as determined by the ELISA, could be explained by the large amount of protein in the sonicates (Table 2). The large amount of protein, most of which was non-toxin protein, resulted in greater competition with Salmonella toxin for binding space in the wells of a polystyrene microtiter plate. The resulting ELISA values for the sonicates were lower than those of the filtrates. There did not appear to be an increase in synthesis of Salmonella toxin due to the presence of MTC. Based on these data, toxin was not released in significant amounts until the cells were lysed in the presence of MTC.

The heat stability study indicated that heating the filtrate containing toxin from Salmonella strain 9630 (MTC) to a temperature above 65°C for 1 hour caused a 50% reduction of its biological effect on CHO cells, but the antigenic structure of the toxin as recognized by the ELISA remained intact. It was only under extreme conditions, such as autoclaving, that the antigenic structure of the molecule was altered. In addition, filtrates and sonicates of five other Salmonella strains (SL 1027, SR11, 9186, 10016, 8994) were each heated by autoclaving for 15 minutes. The biological activity of filtrates and sonicates from the five strains was drastically reduced by 95% after autoclaving as determined by the CHO floating cell assay. This indicated that intracellular Salmonella toxin found in the sonicates was heat labile as well as that found in the filtrates. The antigenic structure of Salmonella toxin in the filtrates of the five Salmonella strains previously mentioned was altered an average of 35% by autoclaving; whereas, sonicates of the same strains experienced a loss of an average of 64% as ascertained by the ELISA. Perhaps the antigenic structure of the Salmonella toxin in the filtrates was less heat sensitive than the sonicates because of an association with non-toxin molecules in the filtrates. This may have had some protective function when the toxin was subjected to heating. Further studies of this phenomenon must be conducted before any definitive explanation can be made. The heat lability of Salmonella toxin found in this study confirmed the previous data obtained by Sandefur and Peterson (17).

Partial purification of Salmonella toxin of crude filtrate from Salmonella strain 9630 grown with MTC was accomplished by gel filtration through a calibrated column of Sephadex G-150 equilibrated with P.D. buffer at pH 7.0. A molecular weight of at least 110,000 daltons for Salmonella toxin was calculated with known protein standards. This molecular weight for the toxin was within an acceptable range of the 90,000 dalton molecular weight of Salmonella toxin calculated by Sandefur and Peterson (17). This toxin was associated with the first small peak of the elution pattern which was near the void volume of the Sephadex G-150 column as determined by the CHO floating cell assay and the ELISA. A more definitive estimate of the molecular weight of Salmonella toxin will be made in the future using other methods.

Preparative flat-bed isoelectric focusing has proven to be extremely useful in terms of isolating the Salmonella toxin and characterizing its isolectric point. The Salmonella toxin was shown to be negatively charged and had an isoelectric point in the pH range of 4.3 to 4.8 as monitored by the ELISA and the CHO floating cell assay. This data coincides precisely with the isoelectric point of Salmonella delayed PF reported by Peterson and Sandefur (16). Preparative isoelectric focusing has the possibility of being used as a final purification step for future work because of its ability to separate

Salmonella toxin from other molecules based on the isoelectric point of the toxin. The protein concentration of fractions containing *Salmonella* toxin was unfortunately too small to be detected as bands by Coomassie Blue G250 stain in polyacrylamide gels. The *Salmonella* toxin was present in ng quantities in filtrates which is below the detectalbe range for stains such as Coomassie Blue. This explains why the antigenic and biologic activity was not associated with a significant protein peak when subjected to preparative isoelectric focusing (Figure 9). In the future, immunochemical experiments using techniques such as rocket and two-dimensional immunoelectrophoresis will be conducted to ascertain purity of the *Salmonella* toxin.

Both the ELISA and CHO floating cell assays were determined to be very sensitive and reliable for the detection of antigenic and biological characteristics of *Salmonella* toxin, respectively. The CHO floating cell assay was capable of detecting as little as 0.01 ng of cholera toxin. The assay was based on the capacity of *V. cholerae* and *E. coli* enterotoxins to increase, the adhesiveness of proliferating CHO cells causing them to accumulate on the adherent monolayers (14). In this study, we determined that *Salmonella* toxin also caused an increase in the adherence of CHO cell monolayers. It was the accumulation of adherent proliferating cells to the monolayer that accounted for the smaller number of CHO floating cells found in the medium. Therefore, CHO cells not treated with cholera toxin or *Salmonella* toxin had a larger number of floating cells than CHO cells treated with either toxin. The CHO floating cell assay proved to be a convenient and reliable tool for measuring the biological activity of both *Salmonella* and cholera toxin.

The absence of biological activity does not always indicate the absence of antigen. With this in mind, the ELISA was utilized to detect the presence of those *Salmonella* toxin antigens shared with cholera toxin in a quantitative manner. The ELISA was capable of detecting quantities of cholera toxin as small as 0.37 ng. Toxin preparations from both *V. cholerae* and *Salmonella* strains were utilized as antigens in the ELISA along with culture media controls. The results indicated that only the antigen preparations of cholera toxin and *Salmonella* toxin in the presence of specifically purified cholera antitoxin were able to elicit a positive ELISA response (O.D. 400.n.m $\geq$ 0.1). Therefore, the specificity of the ELISA was directly related to the specificity of the specifically purified cholera antitoxin. Non-specific adsorption of cholera antitoxin to the microtiter plate wells was prevented by PBS-Tween buffer in which the cholera antitoxin was diluted. The detection of *Salmonella* toxin with the ELISA, utilizing cholera antitoxin, was further evidence for the cross reactivity that exists between cholera toxin and *Salmonella* toxin.

The CHO floating cell assay yielded consistently larger cholera toxin equivalent unit values than those of the ELISA. One possible explanation for the variance in the toxin values between the ELISA and CHO floating cell assay was the competition between the *Salmonella* toxin molecules and other non-toxin molecules for binding space on the surface of the wells of the polystyrene microtiter plate of the ELISA as discussed previously. In the CHO floating cell assay, there is no competition between *Salmonella* toxin and other molecules for the CHO cells. Thus, higher cholera toxin equivalent values were obtained. Another explanation for the variance in the toxin values between the

ELISA and CHO floating cell assay related to the antigenic similarity of Salmonella and cholera toxin. Although similar levels of biological activity of the two toxins exist, they may have only partial homology. This partial cross-reactivity that exists between the two molecules could account for the smaller ELISA values obtained when cholera antitoxin was utilized in the ELISA to detect Salmonella toxin. When pure Salmonella toxin is obtained in the future, Salmonella antitoxin will be prepared and employed in the ELISA.

We have attempted to study some of the biochemical, biological, and antigenic characteristics of Salmonella toxin employing the CHO floating cell assay and the ELISA. The coupling of an antigenic assay with a biological assay was very advantageous in the study of Salmonella toxin. Both the CHO floating cell assay and the ELISA have proven to be very sensitive and reliable. The addition of MTC enhanced the amount of Salmonella toxin present in filtrates but had the reverse effect on sonicates of Salmonella cells. The antigenic structure of Salmonella toxin was relatively heat stable although antigen contained in Salmonella filtrates was more heat stable than antigen in cell sonicates. Heating destroyed virtually all the biological activity of Salmonella toxin in both filtrates and sonicates. The toxin had an isoelectric point in the pH range of 4.3 to 4.8 and an estimated molecular weight of at least 110,000 daltons. With exception of the range for its isoelectric point, molecular weight, and low concentration in filtrates and sonicates, Salmonella toxin appeared to be very similar in biochemical, biological, and antigenic characteristics to cholera toxin which has an isoelectric point near pH 7, a molecular weight of

84,000 daltons, and is produced in relatively large concentrations by some strains. We feel that the antigenic and biological assays described here will provide an effective basis for extending the study of the characteristics, mechanism of toxin release, and role of Salmonella toxin in the pathogenesis of salmonellosis.

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FIGURE LEGEND

Figure 1. Diagramatic scheme of the modification of the CHO floating cell assay.

Figure 2. A standard curve utilizing various concentrations of purified cholera toxin in the CHO floating cell assay. The solid line represents the decreased number of floating cells as the concentration of cholera toxin is increased.

Figure 3. Photomicrographs of fixed and stained normal (left) A and cholera toxin treated cells (right) B of the monolayer after the removal of floating cells. The magnificiation is 500X.

Figure 4. A standard curve utilizing various concentrations of purified cholera toxin in the ELISA. GARA is alkaline phosphatase labeled goat anti-rabbit antibody conjugate.

Figure 5. The effect of mitomycin C on the presence or absence of Salmonella toxin in filtrates and sonicates of Salmonella strains as determined by the ELISA.

Figure 6. The effect of mitomycin C on the presence or absence of Salmonella toxin in filtrates and sonicates on Salmonella strains as monitored by the CHO floating cell assay.

Figure 7. The effect of heat on the biological activity and antigenic structure of Salmonella toxin. A separate aliquot of culture filtrate containing Salmonella toxin was incubated at one of the temperatures in the graph for 1 hour with the exception that incubation at the 100°C temperature was for 10 minutes. The whit- bars represents ELISA values and the shaded bars represent the number of CHO floating cells.

Figure 8. Isoelectric focusing of 1 mg of culture filtrate containing Salmonella toxin in a preparative flatbed with an ampholine range of pH 3.5 to 5. The pH and 280 n.m. absorbancy were plotted against fraction number. ELISA values were measured at an optical density of 400 n.m. and are represented by the dotted line, pH values are represented by the solid line, the protein values are represented by dashed line and the CHO cell activity is represented by the solid bar which is equvalent to an average of 2.36 ng of C.T. eg./ml.

Figure 9. Chromatography of 15X concentrated crude filtrate of Salmonella enteritiditis, strain 9630 (MTC) on Sephadex G-150. The optical density (O.D.) at 280 n.m. is represented by a solid line. The column was equilibrated and eluted with P.D. buffer, pH 7.0. Biological activity and toxin antigen was monitored with the CHO floating cell assay and the ELISA, respectively. Values for both assays were expressed as cholera toxin equivalent units (ng/ml). Biological activity is represented by the dashed line and Salmonella toxin antigen is represented by the dotted line.

Figure 1

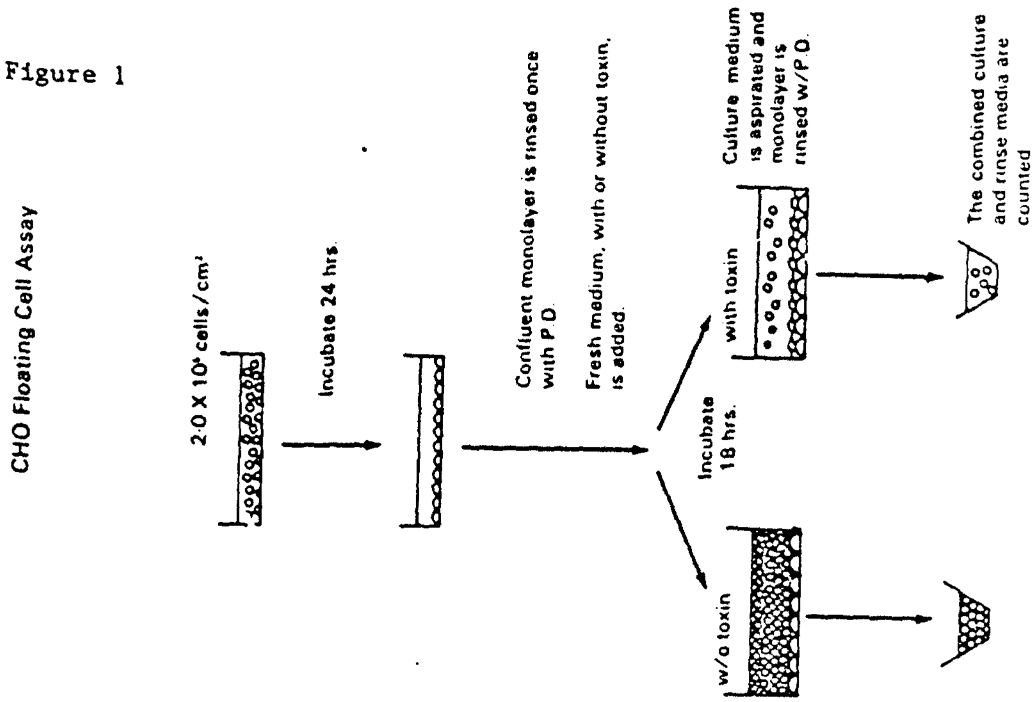


Figure 2

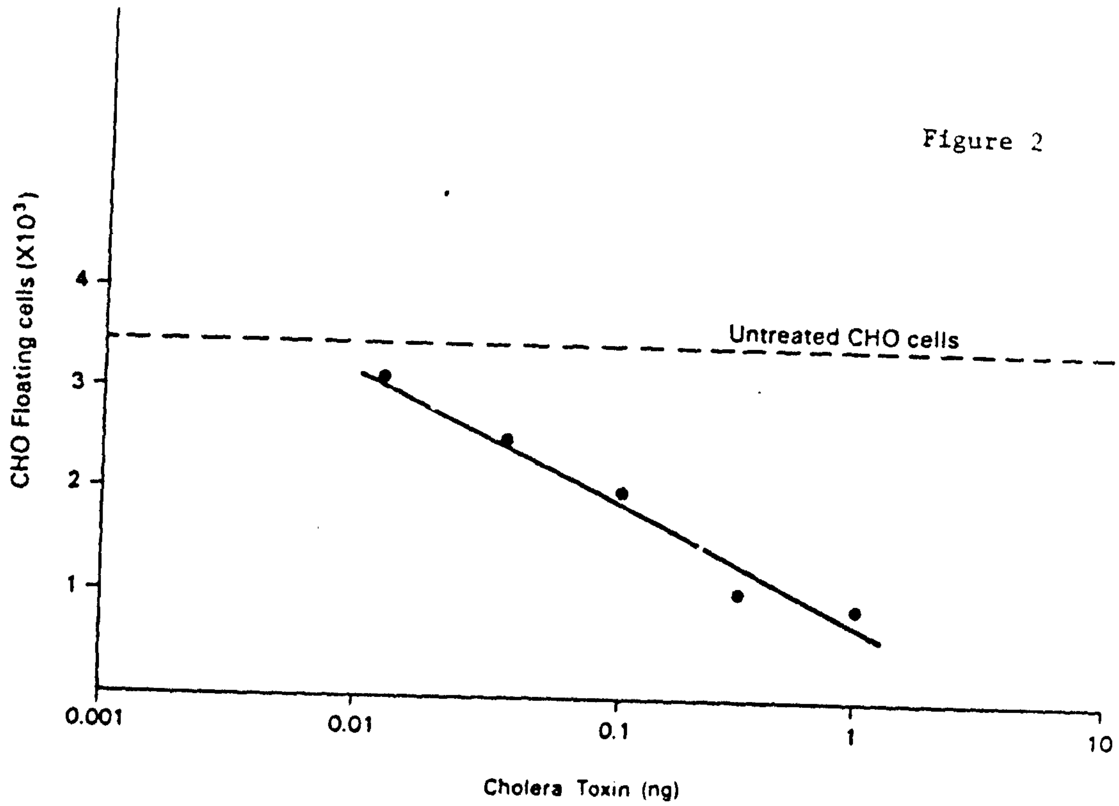
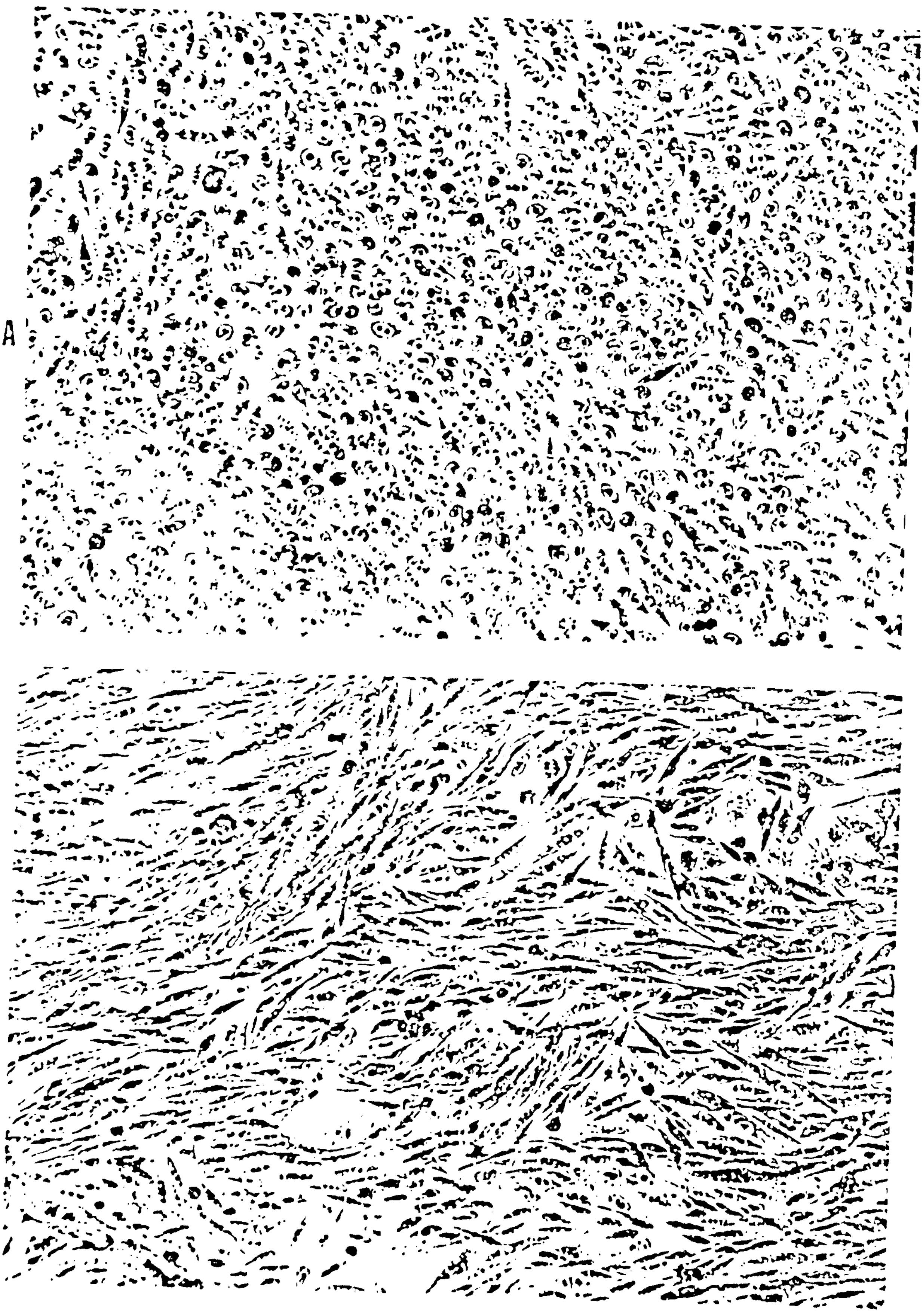


Figure 3

A

B



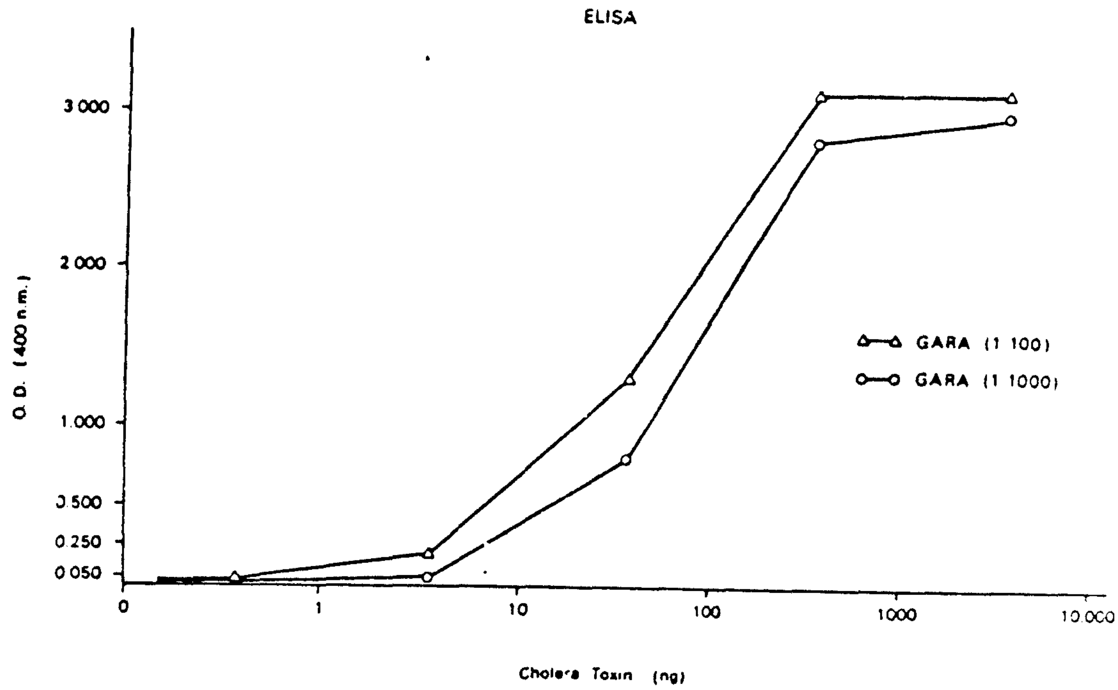


Figure 4

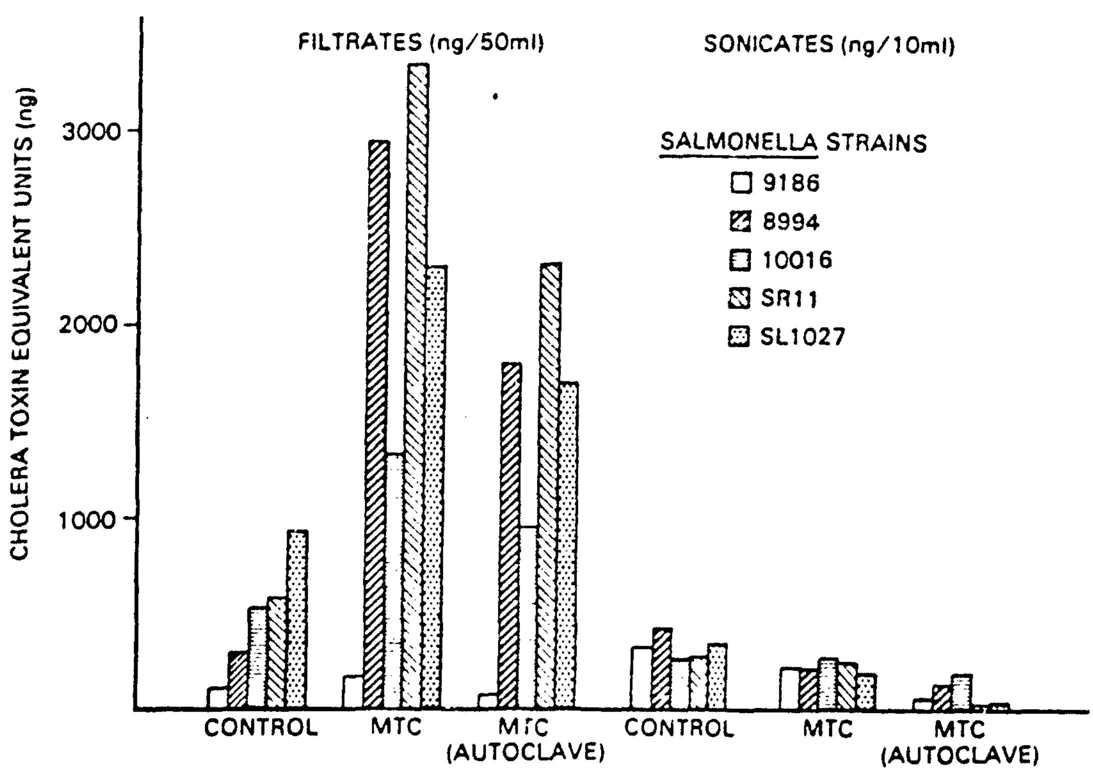


Figure 5

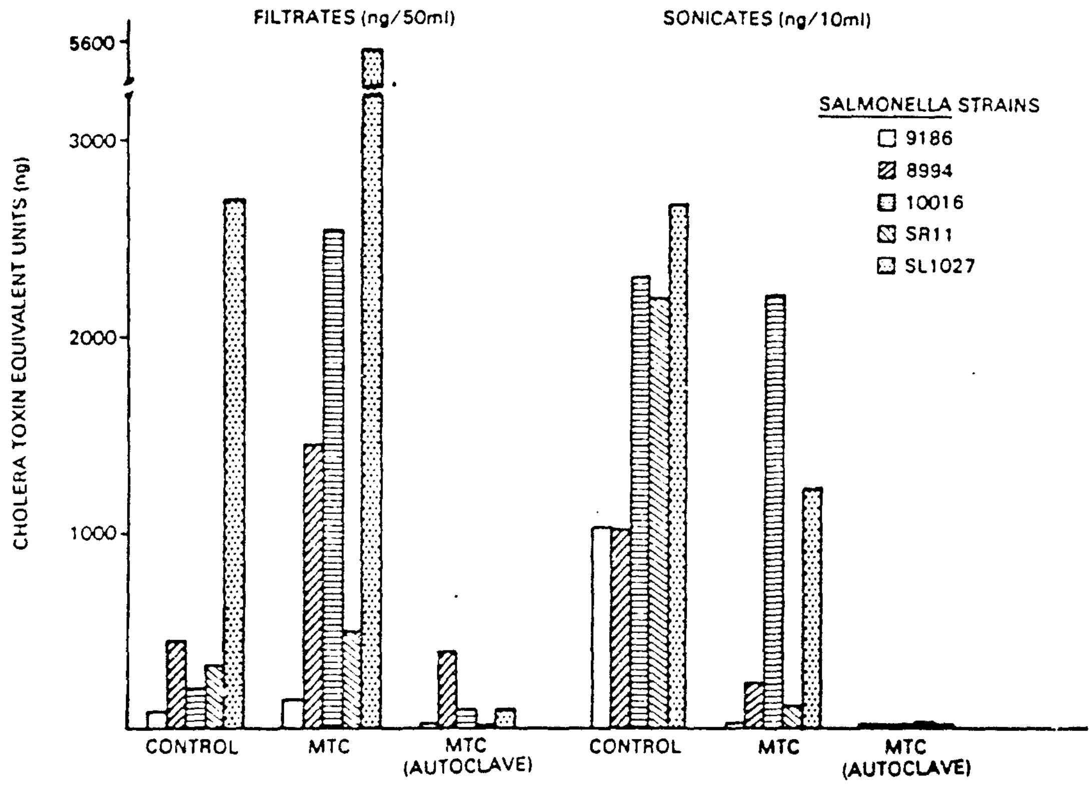
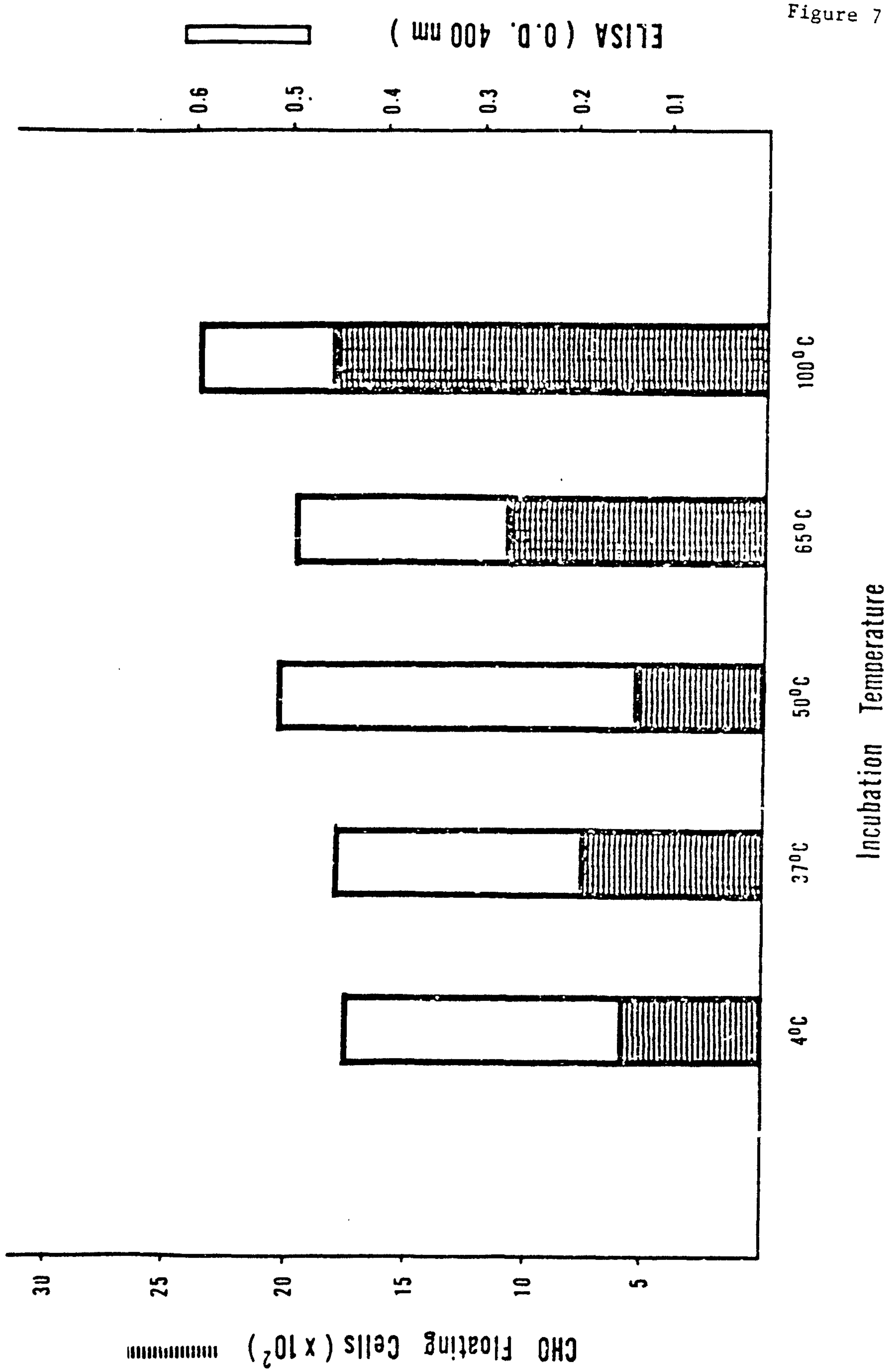

FILTRATES (ng/50ml)
SONICATES (ng/10ml)
5600
3000
2000
1000
CHOLERA TOXIN EQUIVALENT UNITS (ng)
SALMONELLA STRAINS
9186
8994
10016
SR11
SL1027
CONTROL
MTC
MTC (AUTOCLAVE)
CONTROL
MTC
MTC (AUTOCLAVE)

Figure 6

Figure 7



ELISA (O.D. 400 nm)
0.6
0.5
0.4
0.3
0.2
0.1
100°C
65°C
50°C
37°C
4°C
Incubation Temperature
30
25
20
15
10
5
CHO Floating Cells (x 10²)

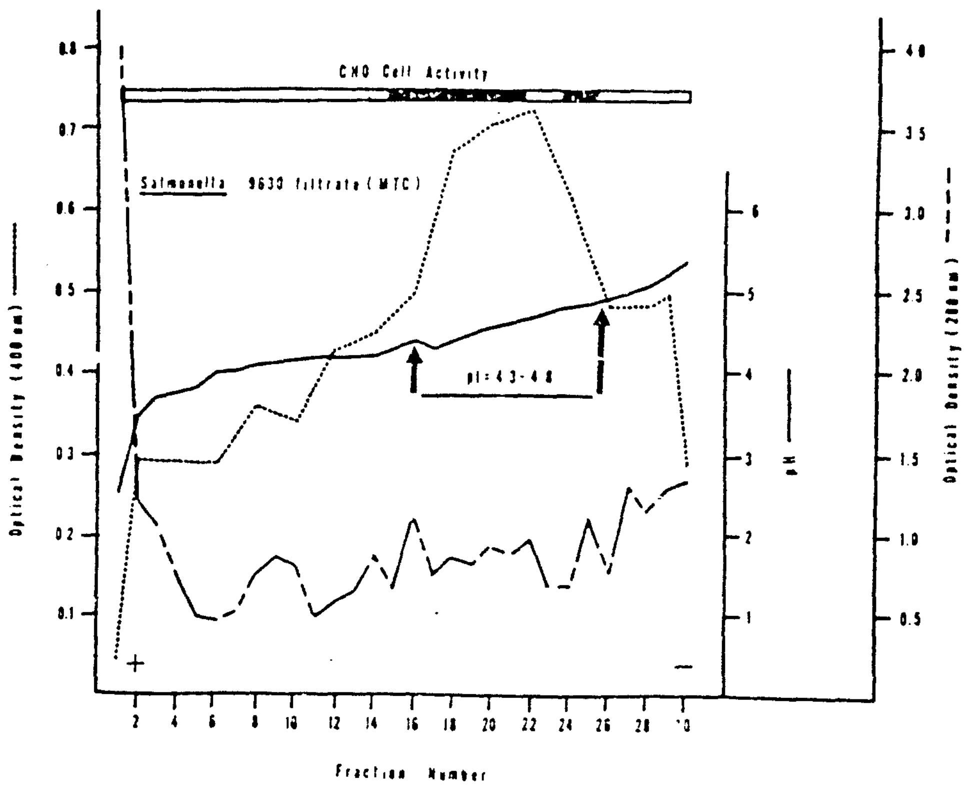


Figure 8

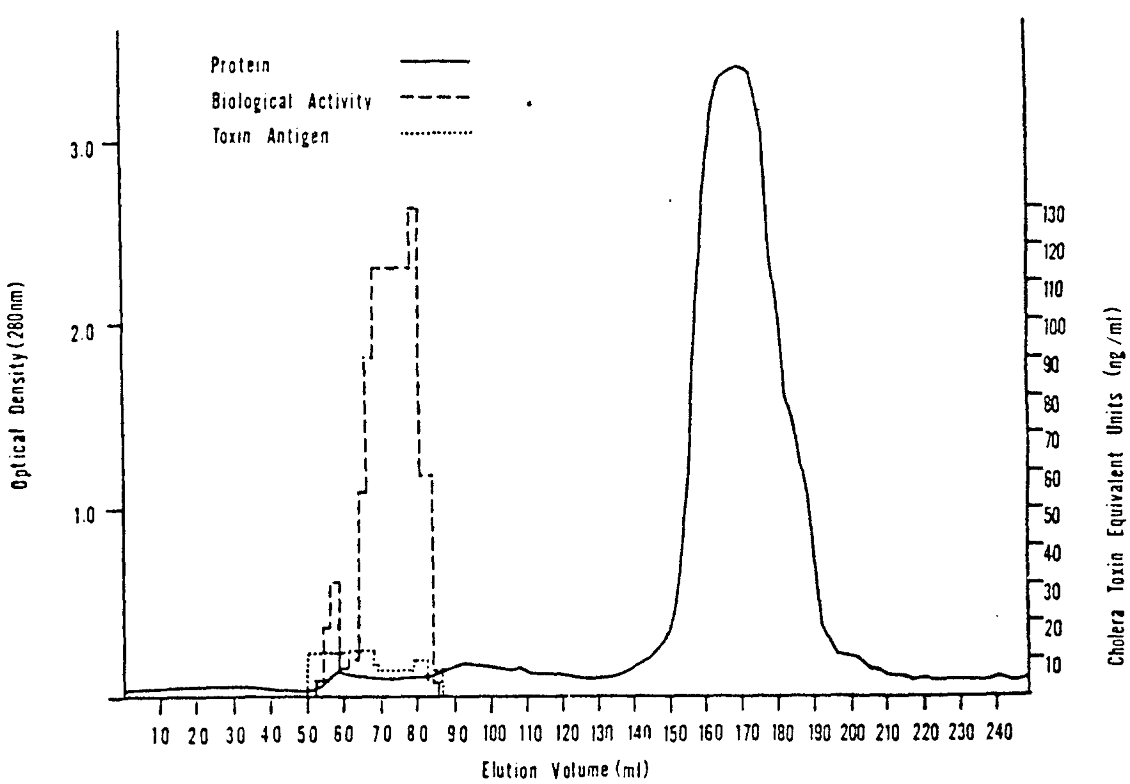


Figure 9

TABLE 1. Specificity of ELISA

Antigen	Optical Density (400 nm)	
	Cholera Antitoxin	Control
Choleragen (20 μg)	3.201	0.070
<u>Salmonella</u> SL1027 filtrate (MTC)	0.285	0.000
SR11 "	0.437	0.004
8994 "	0.337	0.006
<u>Salmonella</u> SL1027 Sonicate	0.155	0.000
" SR11 "	0.322	0.018
" 8994 "	0.226	0.011
CYE medium control	0.012	0.001
CYE-MTC medium control	0.043	0.000

TABLE 2. Bio-Rad Protein Determination

Strains	Filtrates (mg)[b]	Sonicates (mg)[b]
9186	<0.1	8.4
9186 (MTC)[a]	40	<0.1
10016	<0.1	16.9
10016 (MTC)	9	16.3
SL 1027	<0.1	25.7
SL 1027 (MTC)	7.5	0.35
SR 11	<0.1	26.2
SR 11 (MTC)	5	<0.1
8994	<0.1	32.0
8994 (MTC)	4	3.6
medium	<0.1	---
medium (MTC)	<0.1	---

[a]indicates the strains were grown in the presence of mitomycin C (MTC)

[b]total amount of protein

TABLE 3. Net Increase[a] and Decrease[a] due to the Presence of MTC when Compared to the Control Group.

ELISA

	9186	8994	10016	SR11	SL1027	Ave.	
Filtrates	60	2662	700	2675	1375	1494	(net increase)
Sonicates	105	210	+5	30	157	126	(net decrease)

CHO floating cell assay

Filtrates	63	1006	2336	182	2863	1290	(net increase)
Sonicates	108	776	90	2070	1442	1079	(net decrease)

[a]expressed as cholera toxin equivalent units (ng)

TABLE 4. Percentage Loss[a] of Antigenicity and Biology Activity of *Salmonella* toxin in Filtrates and Sonicates of Five Strains of *Salmonella* when Subjected to Autoclaving for 15 minutes.

ELISA

	9186	8994	10016	SR11	SL1027	ave
Filtrates	53%	39%	28%	30%	26%	35%
Sonicates	74%	42%	33%	89%	81%	64%

CHO floating cell assay

	9186	8994	10016	SR11	SL1027	ave
Filtrates	85%	73%	96%	96%	98.2%	90%
Sonicates	---	98.5%	99.5%	99.9%	99.5%	99%

[a]Percentage loss was calculated by dividing the cholera toxin equivalents of (MTC) filtrates by cholera toxin equivalents of (MTC) filtrates subjected to autoclaving for 15 minutes. This percentage value was substrated from 100% and the resulting percentage was assumed to be the percentage loss value.

Factors Affecting Synthesis and Release

of Salmonella Toxin

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Running Head: Salmonella toxin

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Abstract

Several isolates of Salmonella were examined for the capacity to synthesize and release a cholera toxin-like toxin that exerted a biological effect on Chinese hamster ovary cells. The Salmonella toxin, contained in cell sonicates and culture filtrates, was expressed in cholera toxin equivalents (ng), since the CHO cell responses of the two toxins were indistinguishable. Comparative titrations of the Salmonella preparations were also performed using an enzyme-linked immunosorbent assay (ELISA) specific for cholera toxin antigen. The amount of Salmonella toxin synthesized was low (nanogram levels), but was detectable in cell sonicates as early as 6 hours after culture inoculation and reached maximal levels by 12 hours. Salmonella toxin antigen was undetectable in control culture filtrates until 48 hrs, but the addition of mitomycin C at 8.5 hrs resulted in the sudden appearance of toxin antigen at 10-12 hours reaching maximum at 14 hours. A large peak of CHO cell activity was observed at 48 hours in the control culture, but significant CHO cell activity was detected as early as 14 hours. A larger amount of CHO cell reactive material was observed as early as 10 hours in cultures grown with MTC. The mechanism of the MTC mediated phenomenon yielding more toxin in culture filtrates was associated with bacteriophage induction. Bacteriophage plaque assay using a susceptible Salmonella strain revealed free bacteriophage in MTC culture filtrates (but not control filtrates) at 12 hours. Toxin production was greatest when cultures were grown at 30-37°C and lowest at 25°C. The inoculum size and degree of culture aeration (agitation) had little effect on synthesis of the toxin, and comparable toxin production occurred during anaerobic growth.

Introduction

Factors Affecting Synthesis and Release of Salmonella Toxin

Salmonella species produce a heat labile toxin that is similar to cholera toxin in biologic activity and antigenic structure. The *Salmonella* toxin exhibits alterations in vascular permeability in rabbit skin in a manner indistinguishable from that of cholera toxin (14). Similarly, the two toxins elongate Chinese hamster ovary cells (15) and result in increased adherence of dividing CHO cells for the monolayer (9,12). Any of these biologic functions of either toxin can be blocked by incubation of the toxin preparations with monospecific antiserum to purified cholera toxin (12).

Quantitation of *Salmonella* toxin antigen can be accomplished by use of specifically purified antibody to cholera toxin in an enzyme-linked immunosorbent assay (ELISA) developed recently (9). The test measures only those antigenic determinants that are similar to those of cholera toxin.

The amount of *Salmonella* toxin in culture filtrates can be increased by addition of mitomycin C (MTC) to the growing culture (9,11). A similar increase in toxin content of enterotoxinogenic *Escherichia coli* culture filtrates occurred when mitomycin was added (10). The mechanism of this increase in toxin content of *E. coli* culture filtrates was initially believed to involve derepression of a plasmid gene (10), but more recently was attributed to bacteriophage induction (4). The mechanism of this phenomenon in Salmonella was unclear, but prior examination of the toxin content of Salmonella culture filtrates and cell sonicates grown with or without MTC suggested that MTC mediated cell lysis was involved in the increased yields of toxin in culture filtrates (9). The purpose of this study was to determine the earliest stage of Salmonella growth when toxin was detectable and under what cultural conditions toxin production was maximal. The mechanism of the MTC mediated increase in toxin content of Salmonella culture filtrates was further delineated.

Materials and Methods

Organisms. Seven strains of Salmonella typhimurium, 9SR2, LT7, W118-2, TMLR66, SL1027, Thax-1, and M206 were supplied by Dr. Samuel Formal, Walter Reed Army Medical Center. Two additional strains of Salmonella typhimurium, SR11 and RIA, were kindly provided by Dr. L.J. Berry, University of Texas at Austin. Clinical isolates of Salmonella enteritidis strains 6229 and 8994, serotype braenderup, were donated by Dr. T. Huber of the Houston Health Laboratory. Four other clinical isolates of Salmonella enteritidis, which included 9630 serotype newport, 9186 serotype newport, 10016 serotype javiana, and 8832, serotype javiana were also supplied by Dr. T. Huber. Additionally, four clinical isolates of Salmonella enteritidis, which included 986 serotype typhimurium, 2000 serotype typhimurium, 2816 serotype montevideo, and 3774 serotype poona were provided by Dr. Q.T. Box of the Department of Pediatrics, University of Texas Medical at Galveston.

Preparation of cultures for strain survey. Flasks containing 50 ml of Casamino acid-yeast extract (CYE) medium (11) were each inoculated with Salmonella strains from slants of trypticase soy agar. Flasks containing the inoculated medium were incubated with mild shaking (100 r.p.m.) at 37°C for 24 hrs.

Preparation of filtrates. After the incubation period, the cultures were centrifuged at 15,000 Xg for 15 minutes and the supernatants were filtered through 0.22- μm sterile Millipore filter units. The filtrates were placed into sterile plastic tubes for storage at 4°C.

Sonication. The Salmonella cell pellet, which resulted from the previously mentioned centrifugation step, was washed once with 20 ml of sterile phosphate diluent (P.D.) buffer (9) and resuspended in 10 ml of the same buffer prior to sonication (9). Each preparation was submerged in an ice bath and sonicated with a Branson sonifier equipped with a microtip at 70 watts for two separate 3 minute periods. The effect of sonication time was examined using an 18 hour CYE broth culture of Salmonella SL1027. It was centrifuged at 27,000 Xg for 20 minutes, and the cell pellet was resuspended in sterile phosphate diluent (P.D.) buffer to a final volume of 25 ml. The cell suspension was divided into 5 ml aliquots and sonicated for one of the following periods of time: 0, 2.5, 5, and 10 minutes according to the previously stated protocol.

Chinese Hamster Ovary (CHO) floating cell assay. A modification of the CHO floating cell assay devised by Nozawa and coworkers (12) was used in this study (9). Wells of a plastic Linbro multidish were inoculated with 2.0×10^5 CHO cells per cm^2. The cells formed a monolayer after 24 hours incubation at $37^{o}C$ with 5% CO_2. After rinsing the monolayer with 1 ml of P.D. buffer, 0.5 ml of fresh medium with or without 50 µl of Salmonella toxin was added to the wells and the cells were incubated for an additional 18 hours. During this time, proliferating CHO cells, which were not subjected to the toxin, floated off the monolayer into the medium whereas those wells inoculated with toxin contained more cells attached to the monolayer and fewer floating cells. The medium (0.5 ml) containing the floating cells was removed and the monolayer was washed once with 1 ml of P.D. buffer. The medium and the wash were added to accuvettes containing 8.5 ml of P.D. buffer prior to enumeration of floating cells with a Coulter counter. Various concentrations of purified cholera toxin were included in the CHO floating cell assay and biological activity of toxin contained in Salmonella culture filtrates or sonicates was determined and plotted against the responses of the cholera toxin standards. The Salmonella toxin values were expressed as cholera toxin equivalent units (ng) and reflect total biological activity per volume of the culture (50 ml).

Enzyme-linked immunosorbent assay (ELISA). Salmonella toxin has antigenic determinants which are similar to those of cholera toxin (13,15). On the basis of the previously stated observation, the ELISA was employed to detect Salmonella toxin utilizing a specifically purified antibody to cholera toxin. The indirect method of Voller et al. (16) with slight modifications was utilized in this study to detect Salmonella toxin antigen (9). Crude filtrates and/or sonicates containing Salmonella toxin were mixed with an equal volume of carbonate buffer, pH 9.6 a 200 μl of the mixture was allowed to adsorb to the wells of a polystyrene microtiter plate (Cooke) during an overnight incubation at 4°C. The wells were washed with P.B.S.-Tween buffer (pH 7.4) three times for a period of three minutes each. Cholera antitoxin, which was produced in rabbits and specifically purified using a cholera toxin-agarose immunoadsorbent, was added in a 1:50 dilution with P.B.S.-Tween buffer to the wells and allowed to incubate for two hours at room temperature. The wells were washed as previously stated. A 200 μl volume of goat anti-rabbit immunoglobulin conjugated with alkaline phosphatase (Miles Laboratories) was applied in a 1:1,000 dilution of P.B.S.-Tween buffer to the wells and allowed to react with the cholera antitoxin for two hours. Finally, after washing the wells, 200 μl of the colorless substrate p-nitrophenyl phosphate (1 mg/ml in carbonate buffer, pH 9.6) was added and the reaction allowed to progress at 25°C for 30 minutes. The reaction was stopped by the addition of 50 μl of 3M NaOH. Dilutions of purified cholera toxin with carbonate buffer were utilized as standards in the ELISA and Salmonella toxin antigen contained in crude filtrates or sonicates was determined and plotted against the cholera toxin standards. The Salmonella toxin values were expressed as cholera toxin equivalent units and reflect total antigen.

Cyclic AMP assay. One ml of each cell sonicate was added to an equal volume of 10% T.C.A. in 0.1 N HCl; the resulting protein precipitate was removed by centrifugation and the TCA solution was extracted 5 times with 2 ml each of anhydrous ether. After the ether extraction, the samples were lyophilized and subsequently resuspended in 200 μl of 0.05 M sodium acetate buffer (pH 4.0) prior to assay. A modification by Brostrom (2) of the method of Gilman (7) was used to measure cyclic AMP in sonicated cell extracts of selected Salmonella strains.

Quantitation of Salmonella. As an indicator of growth, the turbidity of a 5 ml sample of broth cultures of each Salmonella strain was measured with a Klett-Summerson colorimeter utilizing a number 54 filter. Viable cell counts were determined by the routine dilution plate method.

Bacteriophage titration. A modification of the bacteriophage titration assay of Hershey et al. (8) was employed to detect and quantitate the presence of MTC induced bacteriophage in cell-free culture filtrates of several strains of Salmonella. A 0.5 ml volume of several dilutions of culture filtrate was mixed with 2.0 ml of a 12-24 hour trypticase soy broth (TSB) culture of the susceptible Salmonella strain 9SR2, diluted if necessary to contain not more than $1x10^8$ bacteria per ml. After three to five minutes of incubation at room temperature, 0.5 ml of this mixture was added to 2.5 ml of melted 0.7% agar at 45°C, and the entire contents was poured over a sterile T.S.B. agar plate. The plate thus received 0.1 ml of the bacteriophage preparation. After allowing the soft agar overlay to solidify on a level surface, the plates were incubated without inverting for 24 hours at 30°C. All plaques on the plate were counted with a colony counter.

To determine the presence or absence of bacteriophage in culture filtrates of Salmonella strain SL 1027 during a growth curve experiment, drops of culture filtrate prepared at various times during the experiment were placed on CYE agar plates containing the susceptible Salmonella strain 9SR2. After a 24 hour incubation without inverting at 30°C, the plates were examined for phage lysis.

Protein determination. The protein content of filtrates and sonicates of the Salmonella strains utilized in this study was determined by a Bio-Rad protein assay (1).

Growth curve study. Duplicate 250 ml screw cap flasks, each containing 50 ml of CYE broth, were labeled as to sample incubation time in the following manner: uninoculated control, 0, 2, 4, 6, 8, 10, 12 14, 16, 18, 20, 24, 36, 48, and 72 hours. An overnight culture of Salmonella strain SL 1027 was diluted with sterile C.Y.E. broth to a Klett reading of 170. The culture was further diluted 1:1,000 with sterile CYE broth bringing the concentration to approximately 1.6×10^6 cells per ml. All flasks, with the exception of the uninoculated control were inoculated with 500 μl of the diluted culture. At 8.5 hours after inoculation of the CYE broth, MTC was added to one of each duplicate flask to yield a final concentration of 0.5 μg/ml (11). The appropriate flasks were removed and placed into an ice bath at each of the designated incubation times. A 5 ml aliquot was removed from the culture flasks for measurement of turbidity and another 5 ml aliquot was removed for viable cell counts. The remaining portion of the culture was decanted into appropriately labeled centrifuge tubes and centrifuged at 27,000xg for 15 minutes. After centrifugation, 12 ml of the culture supernatant was sterilized by Millipore filtration. A 10 ml volume of this sterile culture filtrate was stored at 4°C prior to assay. The remaining 2 ml was used for pH measurement. The resulting pellet from the centrifugation of the Salmonella broth culture was suspended in 20 ml of sterile cold P.D. buffer, centrifuged, and resuspended in 10 ml of sterile cold P.D. buffer. The cell suspension was then sonicated and centrifuged at 27,000xg for 15 minutes prior to filter sterilization.

Other cultural conditions. Each of the cultures described in this section was incubated for 14 hours, with or without the addition of MTC as described above, except where otherwise indicated. The effect of incubation temperature on Salmonella toxin production was examined by inoculation of 250 ml screw cap flasks containing 50 ml of CYE broth with 8×10^5 viable Salmonella SL 1027. Each flask was incubated without shaking at 25°C, 30°C, or 37°C. The effect of inoculum size on toxin production was determined by inoculation of similar shaken flasks incubated at 37°C with a series of ten-fold inocula up to 8×10^8 viable Salmonella SL 1027. The effect of culture aeration on toxin production (without MTC) was examined by inoculation of flasks containing 50 ml of CYE broth with 8×10^5 viable Salmonella SL 1027 and incubation at 37°C without shaking. Some of the flasks were incubated in BBL anaerobic jars at 37°C. Prior to inoculation, the medium was rendered free of dissolved oxygen by heating of the medium and incubation of the flask in a BBL anaerobic jar for 24 hours. The Salmonella filtrates and sonicates from each of the above mentioned studies were prepared and assayed as described earlier.

Results

Figure 1 illustrates the efficiency of toxin release from *Salmonella* strain SL1027 after sonication for increasing periods of time. The data indicated that maximum release of *Salmonella* toxin occurred when the cell suspension was sonicated for five minutes. Continuation of the sonication process beyond 5 minutes resulted in a marked decrease in toxin activity. Although sonication was performed in an ice-water bath, the tubes containing the cell suspension often became warm after prolonged sonication. Therefore, heat generated during excessive sonication appeared to inactivate the heat labile toxin. For this reason, subsequent cell suspensions were subjected to two separate three minute sonication periods. Following the first sonication period, the cell suspensions were rechilled in an icebath to minimize the generation of heat. The use of two consecutive three minute sonication periods resulted in the lysis of 99.995% of bacteria in the cell suspension or a drop in cell viability from 6.9×10^9 to 3.3×10^5 cells/ml.

A number of *Salmonella* strains were examined for the capacity to make a heat labile toxin that affected Chinese hamster ovary cells. Table 1 contains a list of *Salmonella* strains, organized in order of decreasing toxin production. The culture opacity and cyclic AMP content of the bacterial cell lysate preparations were generally unrelated to the level of *Salmonella* toxin, as measured by the CHO floating cell assay. The toxin activity of each *Salmonella* cell sonicate preparation was compared to cholera toxin and expressed as equivalent units (ng/ml) (9). Under these conditions, the best yield of toxin was obtained from

Salmonella strain SL1027, which yielded 80 ng/ml of cholera toxin equivalent units in the 10 mls of cell sonicate.

The toxin concentration produced by each Salmonella strain was low, compared to that of many Vibrio cholerae strains (3), and each toxin preparation was destroyed by autoclaving for 15 minutes as previously described (9,11).

Salmonella strain SL1027 was selected for further characterization of toxin production, because it produced as much or more toxin than other strains tested in this study. Figure 2 shows the optical density growth curves of this strain growing in shaken broth cultures of CYE medium at 37°C with and without the addition of mitomycin C at 8.5 hours. The upper curve depicts a classical growth curve, but the lower curve reveals a sharp decline in optical density beginning 3.5 hours after addition of the drug. The other two curves in the figure depict a slight drop in pH at 8-10 hours followed by a steady increase to 8.1 - 8.3. The addition of MTC had little effect on the pH of the culture.

The dramatic effect of MTC on the opacity of the growing cultures of Salmonella SL1027 was confirmed and further explained by viability data shown in Figure 3. Almost a 6 log increase in viable cells occurred in the normal control culture yielding 1.4×10^{10} cells/ml. In contrast, MTC added at 8.5 hours caused a four log drop in viable cells between 10 and 14 hours. This dramatic effect in the MTC culture was counteracted by a progressive secondary increase in the number of viable Salmonella between 14 and 72 hours.

The sudden decrease in optical density and cell viability following the addition of MTC was suggestive of bacteriophage induction. Therefore, filter sterilized samples of culture filtrates, from each time period of the Salmonella SL1027 growth curve, were dropped onto indicator lawns of Salmonella strain 9SR2 in soft agar overlays. Figure 4 shows that the MTC filtrates, beginning at 12 hours, produced areas of clear lysis in the bacterial lawn. No lysis was observed with any of the control culture filtrate samples or with the uninoculated MTC containing culture medium. Assured that the lytic reaction was due to the presence of Salmonella specific bacteriophage induced by MTC, quantitation of the free phage in the MTC culture filtrates was accomplished by bacteriophage plaque assay. Figure 5 reveals the appearance of free Salmonella phage in MTC filtrates at 12 hours. The peak of phage activity observed at 12-24 hours corresponded to the region of minimum viability of the bacterial growth curve. The decrease in phage titer at 36 hours resulted from adsorption of the free phage to a second population of Salmonella arising at 36 hours. The reinfection was followed by an increase in free phage by 72 hours.

Salmonella toxin antigen contained in culture filtrates and cell sonicates was measured by the ELISA, which uses specifically purified, monospecific cholera antitoxin (9). Salmonella toxin antigen was expressed as cholera toxin equivalents in ng/ml based on comparison with purified cholera toxin. The data in Figure 6 indicated that toxin antigen in the control culture (without MTC) was not detectable in culture filtrates until 36-48 hours. In contrast, toxin antigen in MTC filtrates increased sharply at 10-12 hours reaching maximum at 14 hours.

Interestingly, the amount of toxin antigen in the MTC filtrates appeared to decline after 14 hours becoming lowest at 36 hours; however , a second increase in toxin antigen appeared by 48 hours.

The Salmonella toxin antigen, contained in sonicates of control cells (without MTC), was detected as early as 6 hours (Figure 6). Maximum toxin antigen in the cells appeared by 12 hours and remained constant during the remainder of the culture period. A similar increase in toxin antigen occurred in MTC treated cells, but a sudden decline was apparent at 14 hours. A rise in toxin antigen occurred in MTC cells by 36 hours after which it remained constant.

The CHO floating cell assay was also used to assay the same culture samples to assess biological activity. Figure 7 revealed that the biological response curves from the growth curve experiment were quite similar to those derived from estimates of toxin antigen in Figure 6. Unlike the ELISA data, control culture filtrates (without MTC) were shown to contain detectable CHO cell activity as early as 14 hours. This activity reached a maximum level by 16 hours and appeared to deteriorate to a minimum level by 36 hours, followed by another rise and fall. The addition of MTC resulted in an earlier and larger release of biologically active toxin into the culture filtrates than the control culture without MTC. A decline in CHO cell activity in MTC filtrates occurred after 24 hours. The CHO cell activity of the cell sonicates from the growth curve appeared similar to the toxin antigen curves derived from the ELISA.

The growth curve data in Figure 3 indicated that MTC yielded two successive populations of Salmonella cells. The ELISA and CHO cell assay revealed the production of Salmonella toxin during the 72 hour growth period, but it did not indicate if both MTC cell populations were actively producing toxin. Table 2 summarizes a comparative study between colonial isolates obtained from the 8 hr control culture and the 36 hour MTC culture. The data indicated that both isolates were comparable in growth rates, yielding similar culture turbidity and viable cell counts. Both isolates were subject to the MTC mediated decrease in turbidity and viability. Furthermore, MTC elicited a comparable increase in toxin in culture filtrates as determined by the ELISA and CHO cell assay. Likewise, MTC caused a decrease in intracellular toxin in both isolates. In general, there was good correlation between the toxin estimates of the samples as judged by the antigenic and biologic assays, although some variation was noted.

Since both the 8 hour control and 36 hour MTC Salmonella isolates were affected by addition of MTC to the cultures, it appeared that both Salmonella contained temperate phage activated by MTC. Filter-sterilized culture filtrates of each isolate grown with or without MTC, were spotted onto lawns of Salmonella . Zones of clear lysis were observed with the MTC filtrates of the isolate from the 8 hour control culture (no MTC) and the 36 hour MTC culture (data not shown). No lysis occurred with filtrates of either isolate grown without MTC. Uninoculated media containing MTC produced no lysis. Therefore, the two populations of Salmonella appearing in the MTC growth curve were both lysogenic for bacteriophage and both produced Salmonella toxin.

The growth curve experiment was performed at 37°C, but an attempt was made to determine if this temperature was optimal for toxin production. Table 3 summarizes an experiment to assess growth and toxin production by Salmonella SL1027 at 25°C, 30°C, and 37°C. Turbidity and viable cell counts were greatest when cultures were grown at 37°C and lowest at 25°C. Toxin production was poor at 25°C and little effect of MTC was observed. Production of intracellular toxin at 30°C was comparable to that produced at 37°C. The lytic effect of MTC was greater at 37°C than 25°C.

An inoculum size yielding approximately 3-4 x 10^4 CFU/ml in the culture was selected for the growth curve experiment; however, larger inocula of Salmonella SL1027 were examined to determine if this variable affected toxin yield. In general, the inoculum size had little effect on final culture turbidity or viability. Similarly, toxin production was neither enhanced nor diminished by varying the inoculum size. No increase in release of toxin into the culture medium was observed with any of the inocula tested.

All studies of toxin production performed thus far have involved aerobic, shake flask cultures. Table 5 provides information relating to toxin production and growth in still cultures grown aerobically and anaerobically. The toxin concentration of the still cultures was comparable to shaken cultures used earlier in this study indicating that shaking of cultures was not required for maximum toxin production. Likewise, toxin was synthesized in cells growing in anaerobic conditions. The toxin concentration produced anaerobically was equal to or in some instances slightly less than that produced under aerobic conditions.

Discussion

Most samples containing Salmonella toxin were assayed for biological activity using the CHO floating cell assay and for antigen content using the ELISA. Our rationale was that data from the two assays should be mutually supportive; however, the presence of antigen might not necessarily reflect biological potency. In general, we observed good correlation between the ELISA and CHO cell assay. It should be emphasized that expression of Salmonella toxin concentration in terms of cholera toxin equivalents (ng) does not eliminate the possibility that the specific activity of the two toxins may be somewhat different; however, both antigenic and biologic assays were generally comparable in estimating toxin content, suggesting a similarity in biologic potency.

Since Salmonella toxin has been shown previously to be heat labile (9,11,13,14,15), we were conscious of the possibility that heat generated during sonication might inactivate intracellular toxin. The time course curve for sonication (Figure 1) revealed maximum release of toxin after 5 minutes of sonication at 70 watts. Based on this observation, we elected to disrupt all bacterial cell suspensions using two consecutive sonication periods each of 3 minutes duration. By rechilling all cell suspensions after the first sonication period, we felt assured that heat inactivation would be minimized. The latter procedure was effective in killing 99.995% of the bacterial cells.

The data in Table 1 indicated that toxin production by Salmonella isolates was common. No relationship of toxin content in cell sonicates to optical density of the culture was apparent, but toxin concentration seemed to increase in proportion to the total protein concentration of the sonicates. The concentration of cyclic AMP in the sonicates was determined as a precautionary measure to ensure that CHO cell activity was not a function of endogenous bacterial cyclic AMP or cyclic AMP from the yeast extract containing culture medium. No relationship between endogenous cyclic AMP content of the sonicates and level of CHO cell activity was observed. Furthermore, autoclaving for 15 minutes destroyed the CHO cell activity. The heat stable cyclic AMP was present in the C.Y.E. culture medium after autoclaving and varied in concentration from strain to strain after growth of the Salmonella isolates. A maximum of 800 ng of cholera toxin equivalents was synthesized by Salmonella SL 1027 cells from 50 ml of broth culture, but Salmonella strains 3774 and 9186 reproducibly yielded little or no toxin.

It should be pointed out that Salmonella strain SL 1027 is a genetic mutant of Salmonella strain LT-2. Strain SL 1027 has been previously shown to lack the capacity to cause fluid accumulation in intestinal loops of adult rabbits, but does retain the capacity to invade the intestinal mucosa and produce an inflammatory response (5,6). Under the conditions of these experiments, we observed that strain SL 1027 synthesized a heat labile toxin that behaved like an adenylate cyclase stimulating toxin (causing CHO cell responses). Therefore, we presently must conclude that virulence factors, in addition to toxin production, determine the lack of intestinal fluid responses by this strain. We suspect that toxin release from all Salmonella strains may be dependent upon cultural parameters such as medium complexity (F.C.W.

Koo and J.W. Peterson, observations to be published). The presence of serum derived factors in vivo might also have an effect on release of the heat labile, cholera toxin-like toxin from the Salmonella cells.

It is interesting to attempt to relate toxin production to the capacity of selected Salmonella strains to cause fluid accumulation in adult rabbits. According to Giannella et al. (5), Salmonella strains TML R66, W118-2, and M206 elicited a fluid response in intestinal loops of adult rabbits, while LT-7, 9SR2, Thax-1, and SL 1027 did not. In addition, data presented here indicated that all of these strains synthesized the heat labile toxin. While these data could be taken as an argument against the involvement of the toxin in the pathogenesis of salmonellosis, it is just as likely that the pathogenesis of salmonellosis may involve several virulence factors in addition to toxin production. Included among them could be adherence potential, invasive capacity, release of toxin from the bacteria and/or mobility of the bacteria in intestinal mucus. It is likely that possession of any single virulence factor (i.e., toxin production, invasion) would not determine overall virulence and that investigations of several virulence factors will be necessary for a complete understanding of the pathogenesis of salmonellosis.

Salmonella SL 1027 was selected for further study because it ranked with the better toxin producing strains. In addition, the possibility of using a strain, that lacked some degree of animal virulence, as a future "toxin production" strain had some appeal from the standpoint of laboratory safety. CYE broth was selected because it was a simple and inexpensive culture medium, previously shown to yield maximum toxin levels in culture filtrates (11). Figures 2 and 3 revealed that strain SL 1027 exhibited excellent growth and a classic growth curve as reflected by optical density and viable cell counts. In contrast, the addition of MTC, at a previously determined final concentration of 0.5 μg/ml (11), had a dramatic effect on optical density and viability of the growing culture. A 4 log reduction in viability occurred 3.5 hours after addition of the MTC. By 14 hours, optical density had also plummetted to a low level indicating that a significant degree of bacteriolysis had occurred. Upon continued incubation, culture viability and optical density increased, which was indicative of some degree of recovery. Interestingly, the culture pH appeared to be unaffected by the MTC phenomenon, and became alkaline (>pH 8) upon prolonged incubation with or without addition of the drug.

Figures 4 and 5 provided an explanation for the rapid decline in cell density and viability at 12-14 hours in the MTC containing cultures as shown in Figures 2 and 3, respectively. The addition of MTC to growing cultures of Salmonella SL 1027 resulted in the activation of temperate bacteriophage. The peak of free bacteriophage was observed in the culture medium during the period of minimum bacterial cell viability. Apparently, some bacterial cells in the initial stage of the culture were free of temperate bacteriophage and continued to multiply until they eventually became infected by exposure to free bacteriophage in the medium. This caused a reduction in free bacteriophage until 36 hours, after which the bacteriophage levels returned to high levels. The second increase in bacteriophage titer was probably due to bacteriophage maturation and release from the newly infected cells. This second increase in free bacteriophage after 36 hours caused the formation of a plateau in cell viability, but viability was not reduced to levels observed at 12-14 hours. We suspect that many bacteria after 36 hours also contained temperate bacteriophage and multiplied without reinfection by free bacteriophage. To investigate this aspect, an isolate was selected from the 36 hour MTC culture and compared to an isolate from the 8 hour control culture for the effect of reexposure to MTC (Table 2). Both isolates exhibited an MTC mediated reduction in cell viability and an increase in bacteriolysis. Therefore, the data indicate that the MTC culture post 36 hours contained a bacterial population that was still lysogenic for bacteriophage. This population probably accounted for the lack of more extensive lysis of the aging culture. Furthermore, the loss of some of the effectiveness of MTC in inducing bacteriophage at this late stage of the culture may have resulted from chemical breakdown of the drug, binding to nucleic acid or absorption by growing cells.

The emergence of bacteriophage in MTC cultures at 12 hours coincided with the time of appearance of Salmonella toxin in MTC filtrates. Toxin antigen (Figure 6) appeared suddenly in MTC filtrates at 12 hours, while CHO cell activity of the MTC filtrates (Figure 7) was detectable by 10 hours. No toxin antigen appeared in control culture filtrates until 48 hours, and no free bacteriophage was detected in the control cultures. The release of toxin antigen in the control culture could be the result of some degree of autolysis in the aging culture or it could be an active secretory process for this exotoxin. The CHO cell assay revealed that biologically active toxin was apparent in control cultures considerably earlier (at 14 hours) than toxin antigen. This apparent discrepancy could possibly be explained by the 10-fold greater sensitivity of the CHO floating cell assay compared to the indirect ELISA method (9). Alternatively, it is possible that some species of biologically active Salmonella toxin, released from control cells beginning at 14 hours, were not related to cholera toxin in antigenic structure. Thus, they would be detected by the CHO cell assay but not by the ELISA. It is also conceivable that biologically active fragments of the toxin or precursor molecules may not possess a full complement of cholera toxin-like antigenic determinants.

The concentration of Salmonella toxin in culture filtrates or sonicated cell preparations was low, rarely exceeding 30 ng/ml of cholera toxin equivalents in culture filtrates even when MTC was added to the culture medium. Variation of incubation temperature below 37°, increasing the culture inoculum several log values higher than 3×10^4, and incubation of broth cultures under static conditions or anaerobic conditions had little effect on toxin concentration in filtrates or sonicates. Efforts will continue to alter *in vitro* cultural conditions in an effort to enhance toxin yield. Success of future purification studies may depend on a thorough knowledge of growth conditions for synthesis and release of this toxin.

The involvement of bacteriophage in release of toxin was observed after addition of MTC to growing cultures. Generalizations that bacteriophage may be involved in normal release of toxin during

Salmonella infections should not be made. Rather, bacteriophage induced cell lysis seems to explain the laboratory phenomenon--the sudden appearance of toxin in culture filtrates of MTC treated cells. The secretory mechanism of release of this toxin from *Salmonella* during infections remains a mystery just as does release of exotoxins from *Vibrio cholerae* and *Escherichia coli*. However, the amount of *Salmonella* toxin produced *in vivo* has not yet been determined, but may not have to be large in order to produce an effect since the *Salmonella* appear to effectively deliver toxin to the intestinal epithelial cells. Future studies are required to determine the precise role of this toxin in salmonellosis.

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Figure Legends

Figure 1. Efficiency of Salmonella toxin release from Salmonella strain SL 1027 after sonication for increasing periods of time. Quantities of Salmonella toxin were expressed as cholera toxin equivalent units (ng) as determined by the CHO floating cell assay.

Figure 2. Turbidity growth curve of shake flask cultures of Salmonella strain SL 1027 with and without MTC added 8.5 hours after inoculation. A decrease in turbidity was observed after the addition of MTC. The pH was monitored during the course of the growth curve.

Figure 3. Viability growth curve of shake flask cultures of Salmonella strain SL 1027 with and without MTC added 8.5 hours after inoculation. The addition of MTC caused a drop in viable cell counts.

Figure 4. Photograph of plates containing a lawn of the sensitive Salmonella strain 9SR2 onto which drops of cell free culture filtrates from Salmonella strain SL 1027, grown with or without MTC in the medium, were placed. The first appearance of phage lysis was from the 12 hour culture filtrate (MTC) indicating the presence of bacteriophage. MTC was added 8.5 hours after the inoculation of Salmonella strain SL 1027 to the culture medium.

Figure 5. Bacteriophage titration during the growth of Salmonella strain SL 1027. The addition of MTC 8.5 hours after the inoculation of the strain caused the induction of bacteriophage beginning at 12 hours post inoculation.

Figure 6. Detection of Salmonella toxin antigen during the growth of Salmonella strain SL 1027 as determined by the ELISA. Salmonella toxin from filtrates (F) and sonicates (S) of Salmonella strain SL 1027 grown with and without MTC in the broth medium was expressed as cholera toxin equivalent units and reflect total antigen.

Figure 7. Biological activity of Salmonella toxin was monitored by the CHO floating cell assay during the growth of Salmonella strain SL 1027 in broth medium with and without MTC. Biologically active toxin present in filtrates and sonicates was expressed as cholera toxin equivalent units.

Table Legends

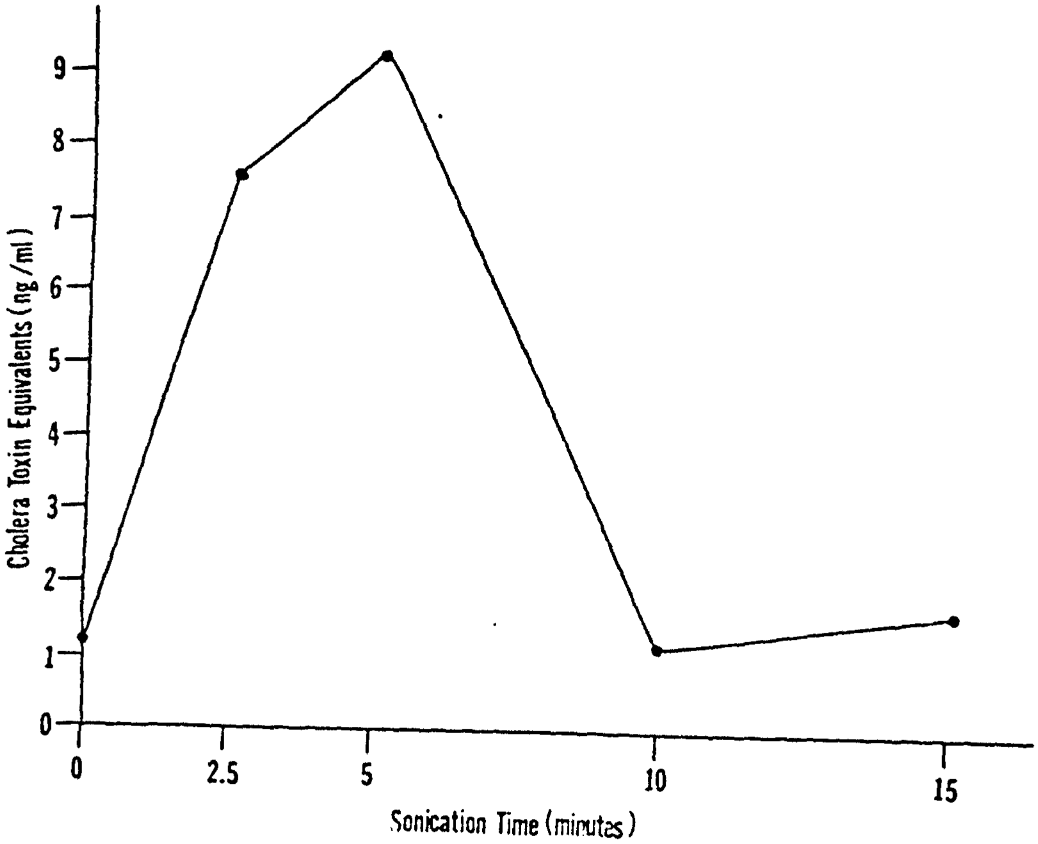
Table 1. Survey of *Salmonella* strains for toxin production, measured by the CHO floating cell assay. Culture opacity was expressed in Klett units. Protein concentration and cyclic AMP levels of sonicated cell preparations were expressed in mg/ml and p moles/ml, respectively.

Table 2. Comparison of growth characteristics and toxin production (measured by CHO cell assay and ELISA) of *Salmonella* isolates (SL 1027) from the control and MTC culture growth curve experiment. Toxin yield of MTC isolate arising in culture after the lytic event and the control culture isolate were similar.

Table 3. Effect of incubation temperature on control and MTC cultures of *Salmonella* SL 1027 grown as stationary cultures.

Table 4. Effect of inoculum size on control and MTC cultures of *Salmonella* SL 1027 grown as shaken cultures.

Table 5. Effect of aeration on toxin production in cultures without MTC. Anaerobic cultures are included for comparison.



Figure

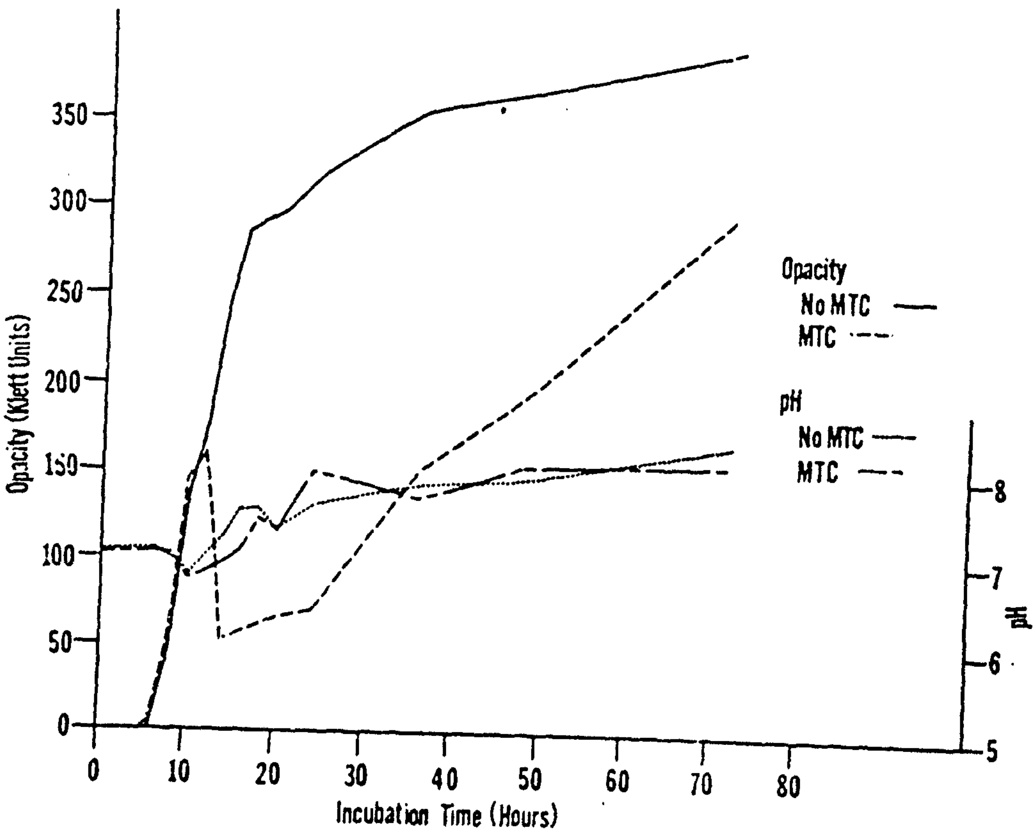


Figure 2

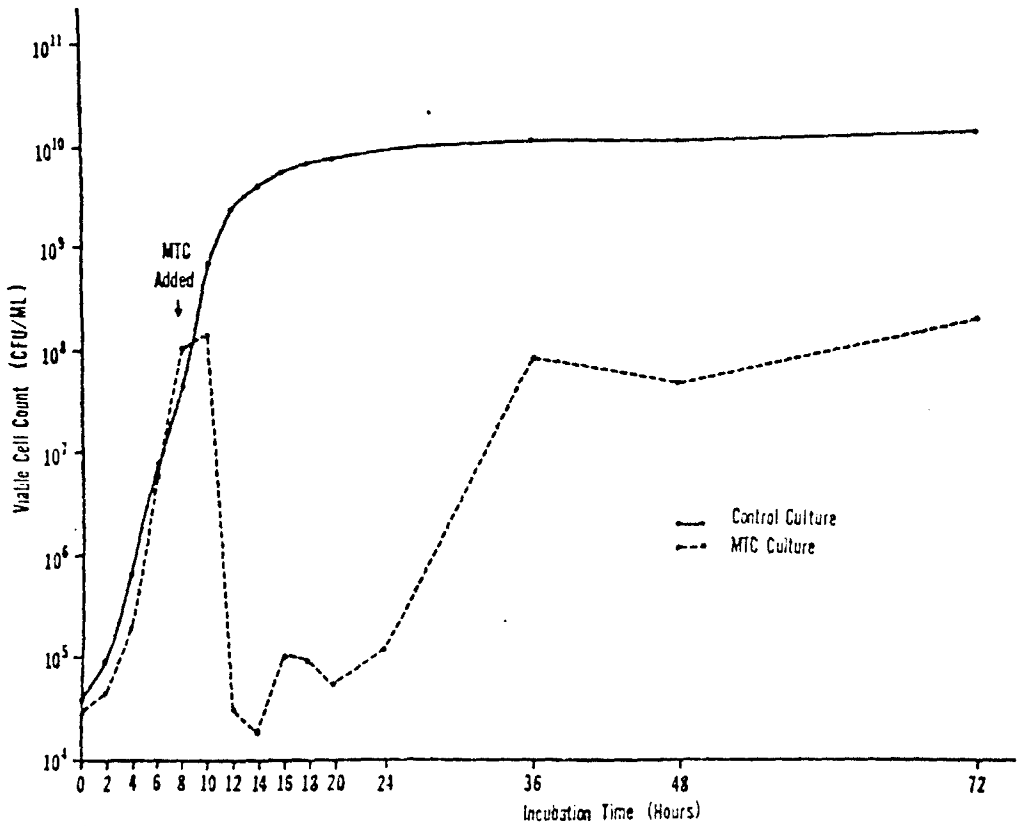


Figure 3

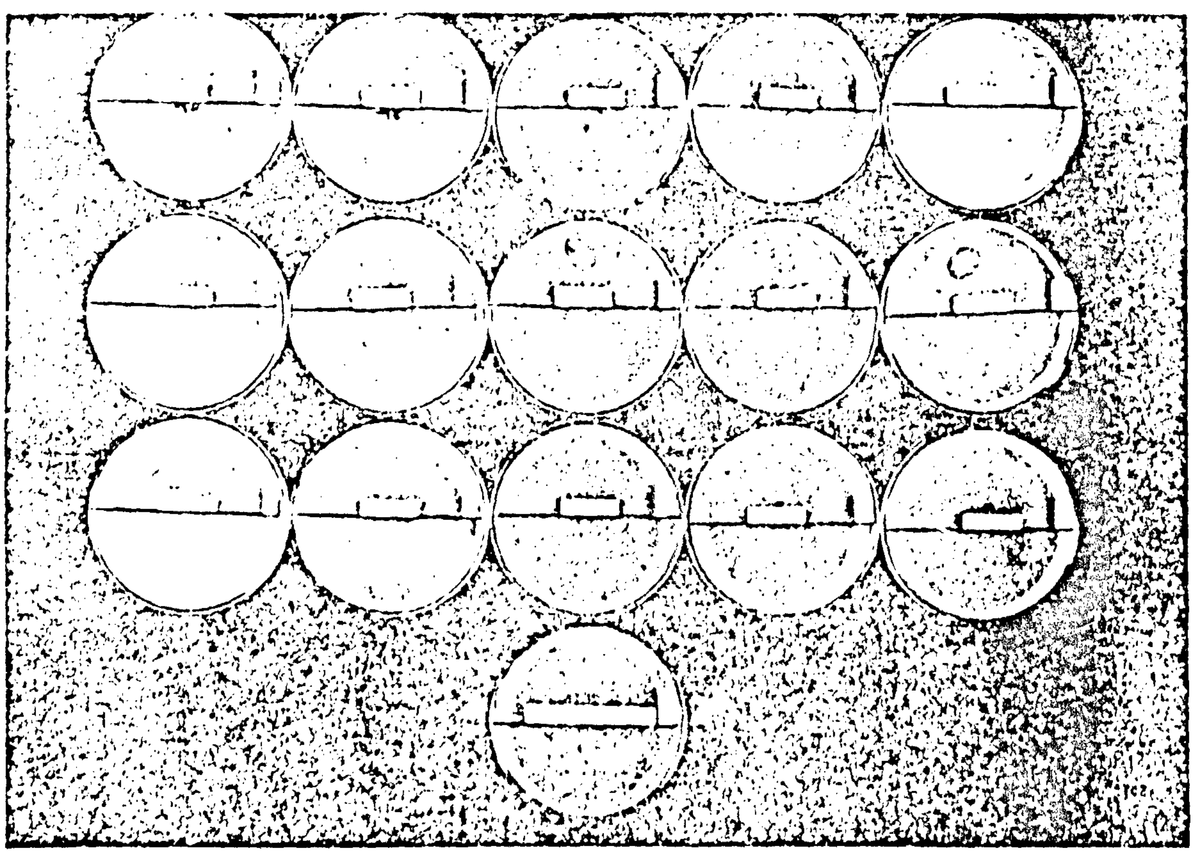


Figure 4

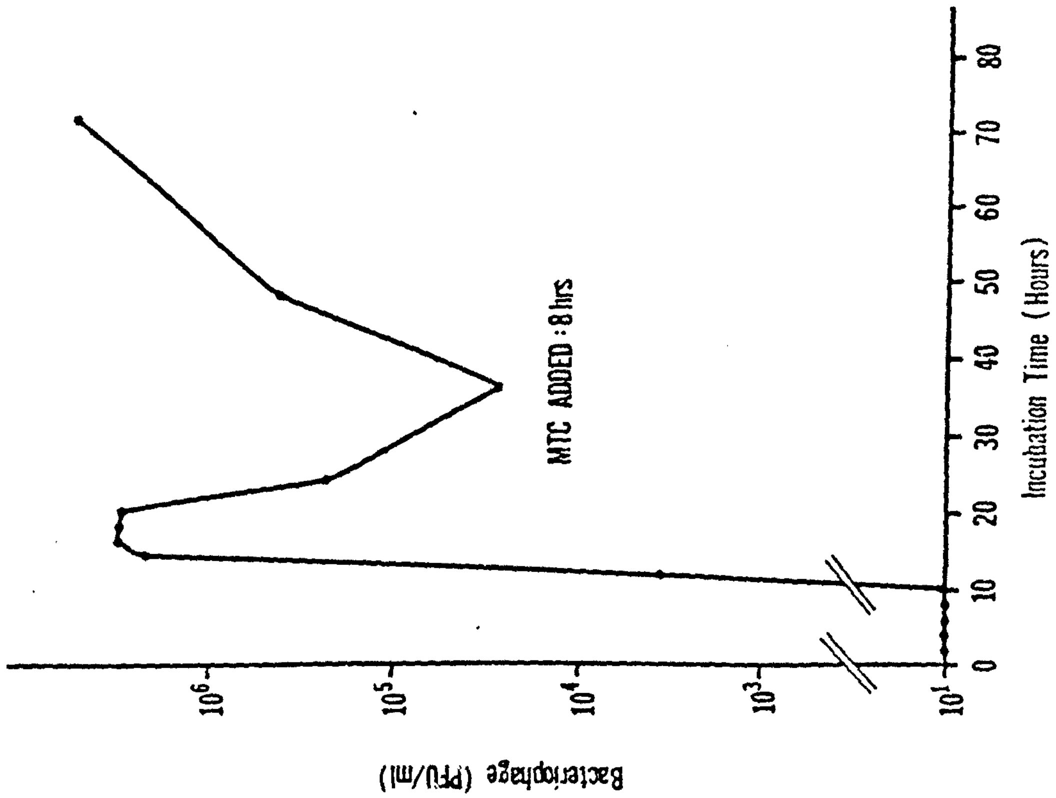


Figure 5

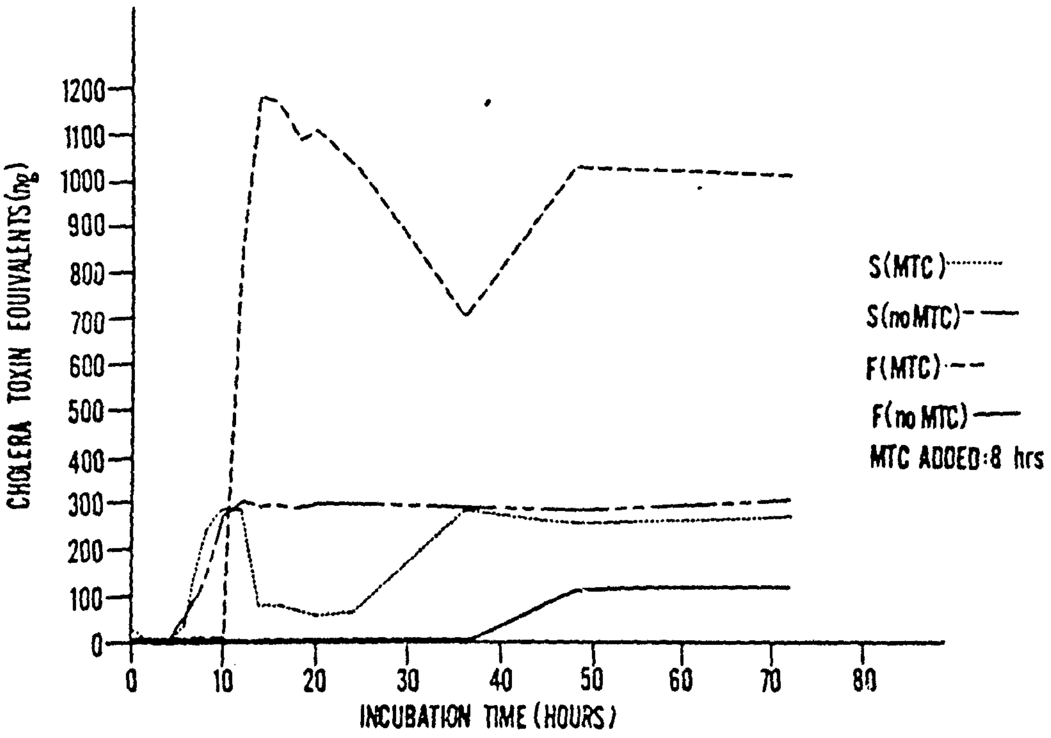


Figure 6

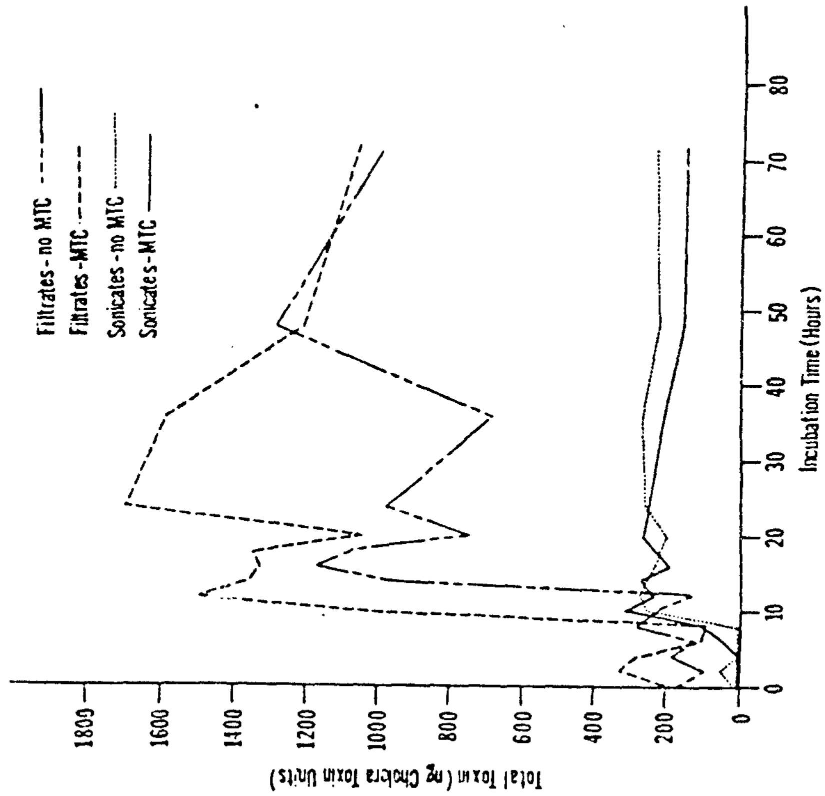


Figure 7

Salmonella Strain	Culture Opacity Klett Units	Cell Sonicates* Protein Conc. mg/ml	Cyclic AMP pmoles/ml	CHO Floating** Cell Activity C.T. eg. ng/ml
SL1027	300	15.0	9,860	80.0
6229	292	4.1	10,915	65.6
10016	325	11.1	2,670	65.0
W118-2	310	11.2	3,690	62.5
2000	266	1.4	1,365	61.4
LT-7	246	4.7	7,575	57.1
8994	294	6.5	5,285	46.2
8832	295	6.8	5,580	43.5
Thax-1	195	2.2	8,540	33.4
RIA	330	6.0	1,400	33.4
THL R66	205	1.4	1,140	29.9
SR11	335	7.6	11,660	26.7
9SR2	182	1.1	5,515	25.7
9630	310	8.6	8,825	20.7
M206	282	2.2	2,900	17.7
3774	275	5.4	2,305	5.8
9186	270	4.0	6,440	3.6
PD-CYE Control	-	0.18	8,205	1.4

* Cells from 50 ml of culture were resuspended in 10 ml of PD prior to sonication.

** Activity was destroyed by autoclaving 15 minutes.

Table 1

Isolate		Klett	CFU/ml	Cells		Filtrates	
				ELISA[a]	CHO[a]	ELISA[a]	CHO[a]
Isolate from 8 hr culture	Control	2[illegible]4	8.5×10^9	248	188	0	164
	MTC	75	3.8×10^5	122	120	775	615
Isolate from 36 hr culture	Control	250	9.4×10^9	238	183	0	324
	MTC	95	8.3×10^6	168	127	875	749

Table 2

[a] Cholera toxin equivalents in total ng from 50 ml of culture

Temperature °C		Klett	CFU/ml	pH	Cells		Filtrates	
					ELISA[a]	CHO[a]	ELISA[a]	CHO[a]
37	Control	156	2.39×10^9	6.79	213	89.6	0	168
	MTC	32	7.1×10^5	6.63	114	128.6	460	638
30	Control	118	1.45×10^9	6.58	230	187.8	0	98
	MTC	48	1.24×10^5	6.59	147	156.4	130	83
25	Control	18.0	6.59×10^7	6.96	137	18.4	0	34
	MTC	14.5	8.8×10^7	7.04	11	6.4	0	44

Table 3

[a] Cholera toxin equivalents in total ng from 50 ml of culture

Inoculum size		Klett	CFU/ml	pH	Cells ELISA[a]	Cells CHO[a]	Filtrates ELISA[a]	Filtrates CHO[a]
$3x10^7$	Control	292	$3.37x10^9$	7.31	225	124	41	731
	MTC	232	$3.10x10^6$	7.16	200	135.8	770	853
$3x10^6$	Control	300	$4.6\ x10^9$	7.43	140	84.2	0	232
	MTC	179	$5.80x10^6$	7.75	237	148.8	840	791
$3x10^5$	Control	290	$4.2\ x10^9$	7.40	185	143	0	243
	MTC	93	$1.25x10^6$	7.45	118	93.6	765	607
$3x10^4$	Control	254	$8.5\ x10^9$	7.10	248	188	0	164
	MTC	75	$3.80x10^5$	6.99	122	120.2	775	615

Table 4

[a]Cholera toxin equivalents in total ng from 50 ml of culture

Time of Harvest in Hours	O_2 variable	Klett	CFU/ml	pH	Cells Elisa[a]	Cells CHO[a]	Filtrates Elisa[a]	Filtrates CHO[a]
14 hrs	Aerobic	166	$3.75\ x10^9$	7.15	960	131.6	210	262
	Anaerobic	153	$1.845x10^9$	6.83	410	130	95	264
20 hrs	Aerobic	198	$3.95\ x10^9$	7.53	662	144.4	260	154
	Anaerobic	186	$3.05\ x10^9$	7.04	842	140.2	60	106
Uninoc. control	Aerobic	0	0	7.10	115	62	0	34

Table 5

[a]Cholera toxin equivalents in total ng from 50 ml of culture

III. Additional Progress (not reported in manuscripts)

A. Nutritional Requirements for Synthesis of Salmonella Toxin.

1. Amino acids requirements. A chemically defined medium, M-9 salts, was selected for the nutritional study since it has been shown by Neidhardt et al. (17) to support the growth of enteric bacteria. M-9 salts contained the following ingredients: KH_2PO_4, Na_2HPO_4, NH_4Cl, $MgSO_4$, $CaCl_2$, NaCl, N-tris(hydroxymethyl)-methyl glycine (tricine), morpholinopropane sulfonate (MOPS), and glucose. The amino acids requirement was identified by placing them into groups of four as described by Callahan and Richardson (2). Each of the 18 amino acids was used at a final concentration of 200 μg/ml, except for phenylalanine and valine (150 μg/ml); isoleucine (100 μg/ml); and tyrosine, tryptophan, and cystine (50 μg/ml).

Salmonella 8994, grown in shake flasks containing M-9 salts and different combinations of amino acids, was harvested after 18 hours of growth at 37°C. The cultures were centrifuged and supernatants were filtered through sterile Millipore filter units (0.20 μm). The cell pellets were washed once with phosphate diluent (P.D.) and resuspended in 10 ml P.D. prior to sonication. Each preparation was sonicated at 65 watts for 5 minutes in an ice-bath (4°C). After centrifugation, the sonicates were removed and filter-sterilized. Both the filtrates and sonicates were tested for the presence of toxin by the CHO floating cells assay.

Results in Table 1 reveal the effect of amino acids on the growth of the culture, protein concentration, and toxin levels of both the filtrates and sonicates of Salmonella 8994 in complex as well as M-9 salts media. As expected, complex media (CYE) appeared superior to M-9 salts medium in the stimulation of cell growth as well as synthesis of toxin. Additionally, sonicates of all cultures contained most of the total protein of Salmonella cells. It is of interest to note that, with the exception of CYE and culture number 5, almost all the detectable toxin was found in the cell sonicates, indicating that Salmonella toxin is largely intracellular in nature when cells are grown in a simple medium. This information on the intracellular distribution of Salmonella toxin will facilitate future attempts in the isolation and purification of the toxin. Clements and Finkelstein (3) have previously shown that most of the E. coli enterotoxin was located intracellularly. We have noticed that the amount of toxin found in culture filtrates is dependent on the type of medium in which the Salmonella are grown.

Generally there is no correlation between cell growth and toxin synthesis in cultures grown in the salts medium. Most groups of amino acids mixtures appeared to stimulate somewhat the synthesis of Salmonella toxin, with groups 2, 4, 5, 8 and 9 having the highest stimulatory effects. The addition of all 18 amino acids in the salts medium caused approximately a six-fold increase in toxin synthesis. A more detailed examination of these data indicated that amino acids that possessed the highest stimulatory effects were: met, gly, cys, his, lys, pro, ser, asp and ala. These findings are quite similar to the amino acids requirements for the synthesis of E. coli enterotoxin (9), i.e., met, gly, his, glu, asp, thr, arg, ser and tyr. In other studies, Liu (15) reported that the synthesis of Pseudomonas exotoxin was

Table 1. Effect of amino acids mixtures on toxin synthesis of _Salmonella_ 8994

Culture number	Content	Klett unit	Filtrate		Sonicate		Total toxin activity[c]
			Protein[a]	Toxin activity[b]	Protein	Toxin activity	
1.	His, Phe, Glu, Arg	244	0.21	0	1.27	38.57	38.57
2.	Gly, Leu, Tyr, Ser	202	0.20	0	1.10	62.49	62.49
3.	Cys, Ile, Trp, Ala	194	0.20	0	1.21	45.02	45.02
4.	Met, Val, Thr, Asp	222	0.21	0	1.37	67.60	67.60
5.	His, Gly, Cys, Met	184	0.21	66.57	1.28	27.59	94.16
6.	Phe, Leu, Ile, Val	204	0.20	0	1.28	34.92	34.92
7.	Glu, Tyr, Trp, Thr	222	0.20	0	1.35	36.59	36.59
8.	Arg, Ser, Ala, Asp	222	0.21	0	0.90	52.74	52.74
9.	Pro, Lys, Ala, Ile	199	0.21	0	0.86	67.97	67.97
10.	M-9 salts	206	0.21	3.13	1.41	23.44	26.57
11.	M-9 salts + 18 amino acids	216	0.24	0	1.10	158.92	158.92
12.	CYE	310	0.22	331.88	9.24	183.15	515.03
13.	TSB + YE	282	0.23	5.58	18.62	175.59	181.17

a, protein concentration in mg/ml.
b, total toxin in nanograms of cholera toxin equivalent units.
c, total toxin in filtrate and sonicate in nanograms of cholera toxin equivalent units.

enhanced when the culture medium was supplemented with ala, asp, glu (15), whereas Callahan et al. (2) had shown that glu, asp and ser were needed for increased production of cholera toxin.

Amino acids can be divided into three groups according to the presence of (i) nonpolar R groups (ala, val, leu, ile, pro, phe, trp, met), (ii) uncharged polar R groups (gly, ser, thr, cys, tyr, asn, gln), and (iii) charged polar R groups (asp, glu, his, lys, arg). In order to verify the precise nature of the previously mentioned amino acids, each of the above was evaluated for its stimulatory or inhibitory effect on toxin synthesis. As shown in Fig. 1 (A,B,C), amino acids that possessed strong stimulatory effects were those that contained polar R groups. With the exceptions of phe and leu, amino acids that consisted of nonpolar R groups were moderately stimulatory. These data, however, are in contrast to the results presented earlier in that stimulatory effect was not dependent on the presence of polar R groups in amino acids. The likely explanation to this discrepancy could be due to the fact that initial parent metabolites for the biosynthesis of other amino acids invariably were amino acids having polar R groups. As a result, the parent compounds could make available all the different types of amino acids which may then be utilized for the synthesis of all necessary enzymes needed for the production of Salmonella toxin.

Effect of different carbon sources on toxin synthesis. Glucose has been employed as a common carbon source in many nutritional studies. It is of interest to know if other carbon sources would also stimulate toxin synthesis in Salmonella. The results, as shown in Table 2, indicated that cells grown in the presence of glycerol contained the highest amount of both intracellular and extracellular toxin. In contrast, glucose was observed to be least stimulatory as compared with all other carbon sources tested. In order to ascertain this antagonistic effect of glucose, toxin levels in filtrates and sonicates were subsequently determined by the ELISA assay (12). As expected, this antigenic assay revealed that glycerol and glucose were highly and least stimulatory, respectively (data not shown). In addition, sonicates of the above were heated at 100°C for 15 minutes. Results in Fig. 2 show that toxin activity in all sonicates was virtually destroyed, indicating that the toxin is heat-labile.

Vitamins and metal cation requirements. Since complex media, commonly used for culturing of bacteria, inevitably contained many unknown growth factors, it is necessary to determine which vitamins play a role in the synthesis of Salmonella toxin. An approximate two-fold increase in toxin activity was observed in cultures supplemented with biotin (Table 3a). Generally, all other vitamins were either non-stimulatory or inhibitory for toxin synthesis. Cultures that contained all six vitamins did not stimulate toxin production substantially, which may be due to the presence of vitamins belonging to the inhibitory group. Table 3b summarizes an experiment to determine the effect of metal cations on production of Salmonella toxin. Enhancement of toxin synthesis occurred when Mg^{++}, Mn^{++}, Cu^{++}, Zn^{++}, or Ni^{++} was added, but the concentration of each appeared to be important.

B. Intestinal Cyclic AMP Responses of Adult Rabbits to Intestinal Challenge with Live Salmonella and Salmonella Culture Filtrates.

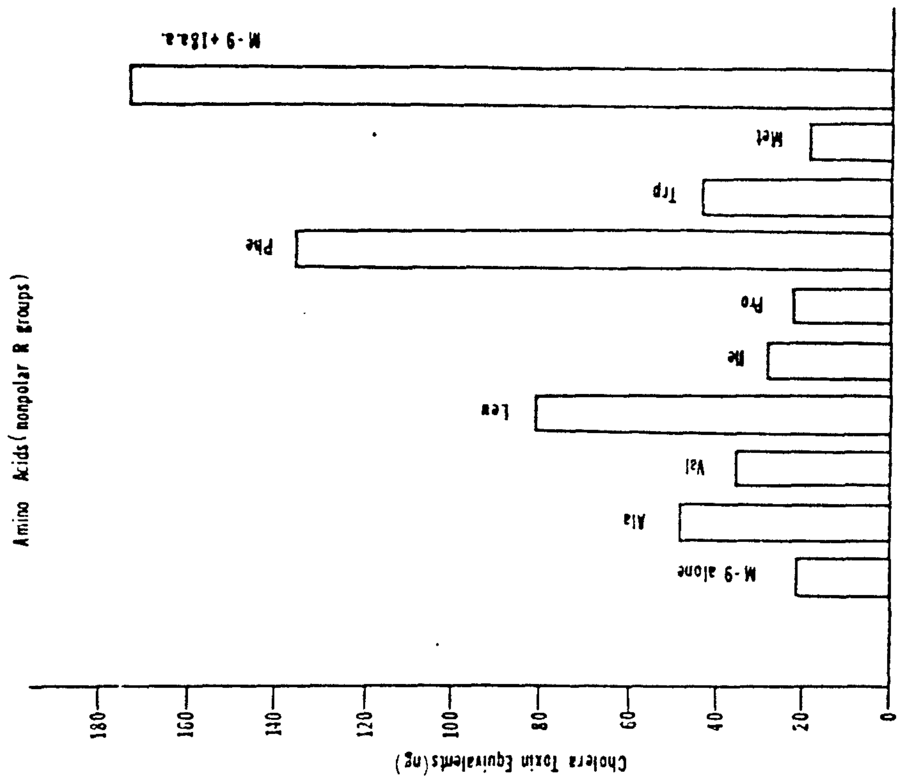


Figure 1

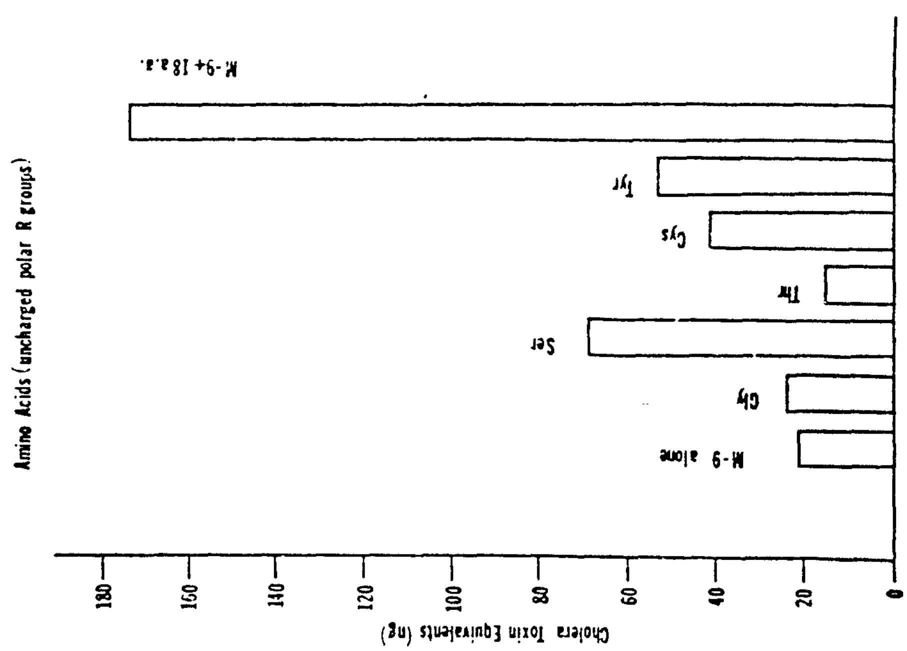


Figure 1

Figure

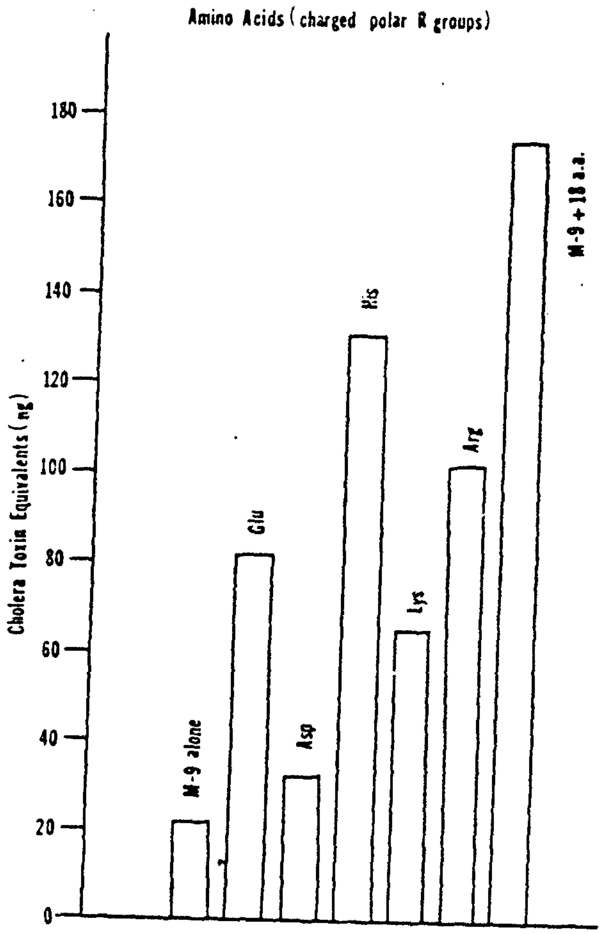

Amino Acids (charged polar R groups)
Cholera Toxin Equivalents (ng)
180
160
140
120
100
80
60
40
20
0
M-9 alone
Glu
Asp
His
Lys
Arg
M-9 + 18 a.a.

Table 2. Effect of carbon source on toxin synthesis of *Salmonella* 8994

Culture number	Content	Klett unit	Filtrate		Sonicate		Total toxin activity[c]
			Protein[a]	Toxin activity[b]	Protein	Toxin activity	
1.	Glucose	230	0.21	15.3	2.13	31.72	47.02
2.	Galactose	202	0.20	0	1.83	77.21	77.21
3.	Glycerol	195	0.21	45.1	1.70	86.35	131.45
4.	Citrate	184	0.20	0	1.44	77.28	77.28
5.	D-Mannose	247	0.20	0	1.54	65.45	65.45
6.	Maltose	242	0.20	0	4.15	83.89	83.89
7.	Lactose	0	0	0	0	0	0
8.	Sucrose	0	0	0	0	0	0

a, see legend to Table
b, "
c, "

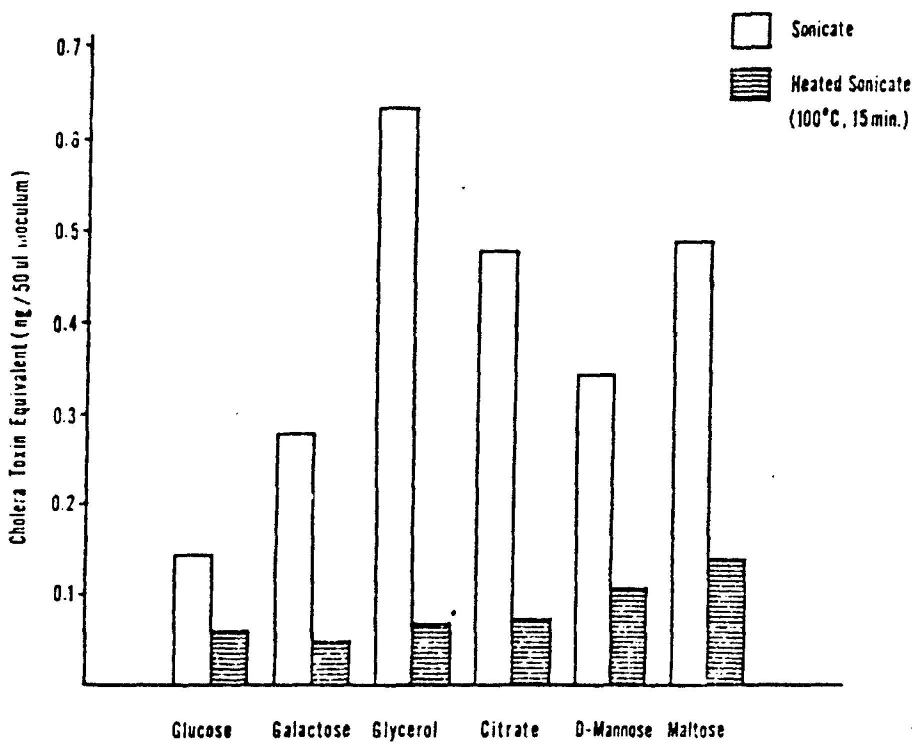

Sonicate
Heated Sonicate
(100°C, 15 min.)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
Cholera Toxin Equivalent (ng/50 ul inoculum)
Glucose
Galactose
Glycerol
Citrate
D-Mannose
Maltose

Figure 2

Table 3a. Vitamins requirement of Salmonella 8994 on toxin synthesis

Culture number	Content	Klett unit	Filtrate		Sonicate		Total toxin activity[c]
			Protein[a]	Toxin activity[b]	Protein	Toxin activity	
1.	Biotin	240	0.20	32.50	1.98	54.37	86.87
2.	Riboflavin	239	0.22	53.67	1.69	4.88	58.55
3.	Thiamine	240	0.20	17.10	2.02	20.43	37.53
4.	Pantothenate	242	0.20	10.60	2.23	15.70	26.30
5.	Folic acid	236	0.19	12.13	2.06	14.51	26.64
6.	Niacin	237	0.21	30.57	1.93	12.52	43.09
7.	All vitamins	243	0.19	8.00	2.36	46.67	54.67
8.	M-9 salts only	234	0.19	23.17	1.90	21.87	45.04

a, see legend to Table
b, "
c, "

Table 3b. Effect of metal cations on toxin synthesis of _Salmonella_ 8994

Culture number	Content[a]	Klett unit	Filtrate		Sonicate		Total toxin activity[d]
			Protein[b]	Toxin activity[c]	Protein	Toxin activity	
1.	Cu^{++} (high)	210	0.19	6.10	1.81	23.37	29.47
2.	Cu^{++} (low)	210	0.20	31.91	1.85	29.00	60.91
3.	Zn^{++} (high)	206	0.19	19.21	2.03	45.93	65.14
4.	Zn^{++} (low)	208	0.31	16.66	2.06	19.02	35.68
5.	Fe^{++} (high)	230	0.19	4.70	1.62	30.34	35.04
6.	Fe^{++} (low)	216	0.20	2.30	1.88	30.63	32.93
7.	Mg^{++} (high)	207	0.19	22.81	2.11	55.90	78.71
8.	Mg^{++} (low)	206	0.20	12.60	1.75	56.62	69.22
9.	Mn^{++} (high)	230	0.19	80.72	2.18	38.91	119.63
10.	Mn^{++} (low)	220	0.20	15.41	2.00	31.11	46.52
11.	Ni^{++} (high)	220	0.21	17.36	1.72	41.75	59.11
12.	Ni^{++} (low)	203	0.19	1.25	1.91	29.75	31.00
13.	M-9 salts only	210	0.19	19.60	1.95	14.26	33.86
14.	M-9 salts + all cations (high)	216	0.19	64.16	2.47	49.62	113.78
15.	M-9 salts + all cations (low)	222	0.20	16.14	1.59	54.25	70.39

a, high = 50ug/ml; low = 5ug/ml (all at final concentration)
b, c, d, see legend to Table

We began the study of Salmonella mediated pathogenesis after examining the innovative observations of Giannella et al. (6,7). These investigators discovered that Salmonella strains causing fluid accumulation in rabbit intestinal loops also caused significant increases in intestinal tissue cyclic AMP. Salmonella SL 1027 was a genetically marked LT-2 strain which invaded rabbit ileum, but failed to elicit fluid and did not elevate intestinal cyclic AMP. Substantial data was provided that argued against inflammation as a cause of the elevated cyclic AMP levels. First, the influx of PMN's into intestinal tissue with fluid producing Salmonella strains was insufficient to account for the large increase in cyclic AMP. Secondly, strain SL 1027 was invasive and elicited an inflammatory reaction, but no fluid or cyclic AMP increases were observed. Indomethacin depressed the Salmonella mediated fluid response as well as the cyclic AMP rise, which would be suggestive of involvement of the inflammatory response. However, upon close inspection, indomethacin significantly decreased the intestinal fluid response to cholera toxin (1.68 ml/cm $\longrightarrow$ 0.68 mg/cm). No effect was observed on the cyclic AMP level in cholera toxin loops, while Salmonella infected loops showed a significant decline (468 p moles/mg $\longrightarrow$ 310 p moles/mg). These effects could vary depending on dose of cholera toxin and indomethacin.

We first attempted to substantiate a portion of these data as shown in Table 4. The data indicate that Salmonella SR11 elicited a fluid accumulation response in each of the four rabbits in a manner comparable to TML. Again Salmonella SL 1027 failed to cause fluid accumulation as reported previously. The fluid accumulation response of the loops to Salmonella SR11 was accompanied by an approximate 10 fold increase in mucosal tissue cyclic AMP compared to the 2 fold rise reported for TML. The tissue from rabbit number two may have been incorrectly taken. Since the above data support the previous observation by Giannella et al. (7) regarding elevation of cyclic AMP, we plan additional in vivo experiments as described in the attached proposal to elucidate the mechanism of cyclic AMP elevation and its relationship to the pathogenesis of salmonellosis.

Recently, we prepared 30x concentrates of Salmonella CYE culture filtrates by dialysis of the filtrates against carbowax. When 2 ml volumes were injected into the intestinal lumen of adult rabbit loops, no fluid accumulation was observed. These negative findings continue to be discouraging, but probably reflect the low concentration of Salmonella toxin in the filtrates. We have observed positive fluid responses with partially purified preparations of delayed PF, and these were reported in a manuscript in the last progress report. This manuscript has not yet been published because of a reviewer's criticism that a dose response curve was not included; however, only limited amounts of Salmonella toxin was available at that time for those studies. We are convinced that the data reported are valid, since loop activity could be blocked by monospecific cholera antitoxin. We will strive to substantiate these data and publish the data.

Table 4

Intestinal Loop Challenged with Salmonella strain	Mucosal Cyclic AMP (pmols/mg) Rabbit 1	Rabbit 2	Rabbit 3	Rabbit 4
SR 11	153	15	422	347
SL 1027	14	15	34	29
Proximal Control	13	12	32	17

Intestinal Loop Challenged with Salmonella strain	Fluid Accumulation (ml/cm) Rabbit 1	Rabbit 2	Rabbit 3	Rabbit 4
SR 11	0.87	1.9	2.1	1.6
SL 1027	0	0	0	0
Proximal Control	0	0	0	0

C. Stimulation of Adenylate Cyclase and Elevation of Cyclic AMP Levels by Salmonella filtrates

1. Elevation of cyclic AMP levels in intact Henle intestinal epithelial cells.

Our observation that Salmonella toxin causes elongation of Chinese hamster ovary cells indicated to us that this toxin, like cholera toxin, acts through an increase in cyclic AMP levels. However, we had previously not directly demonstrated that Salmonella toxin treated eukaryotic cells have increased cyclic AMP levels. We have now shown this to be the case. Several strains of Salmonella were grown in HMEM containing 2% fetal calf serum. The cultures were brought to neutral pH and filter sterilized. Monolayers of Henle intestinal epithelial cells were then incubated in these filtrates at 37°C. After 4 hours of incubation, the monolayers were washed twice in PBS and collected for cAMP and protein determinations as described by Guerrant (10). As shown in Table 5, filtrates of strains TML and W118-2 increased cAMP levels in the cells while filtrates of strains M206 and SL 1027 did not. Giannella et al., reported that strains TML, W118 and M206 caused fluid accumulation in rabbit ileal loops, while strain SL 1027 invaded the intestinal epithelium, but did not cause fluid accumulation (6). Thus, this experiment shows some correlation between the ability of Salmonella strains to induce fluid accumulation in vivo and their ability to increase cAMP levels in eukaryotic cells cultured in vitro. The serum enriched medium used in this experiment for cultivation (of the Salmonella) may determine whether toxin is released by these isolates or not, since each of these strains synthesizes the heat labile toxin in CYE broth.

2. Stimulation of adenylate cyclase in pigeon erythrocyte lysates.

In order to demonstrate that the increase in cyclic AMP levels was due to activation of adenylate cyclase, we tested Salmonella filtrates in the pigeon erythrocyte lysate assay (8,9). As described in the previous section, the Salmonella strains were grown in HMEM containing 2% fetal calf serum. In this medium, the bacteria release increased amounts of toxin even in the absence of mitomycin C. After 24 hours of growth, the cultures were centrifuged and filter sterilized. Samples of the filtrates were then tested in the PEL assay. The results of two separate experiments are shown in Table 6. Each value is the average of two separate samples. Adenylate cyclase was stimulated by most of the Salmonella filtrates. Furthermore, the adenylate cyclase activating activity was heat-labile (data not shown).

Thus, we have concluded from these experiments that Salmonella toxin does increase cyclic AMP levels in eukaryotic cells. As in the case of cholera toxin, the increased cyclic AMP levels caused by Salmonella toxin are mediated by an increase in adenylate cyclase activity.

Table 5

Increased cyclic AMP levels in Henle intestinal epithelial cells incubated with Salmonella filtrates

Filtrate from:	pmoles cAMP/mg protein in Henle cells
uninoculated medium	32.7
TML-R66	190.6
W118-2	327.5
M206	9.4
SL 1027	46.6

Table 6

Stimulation of adenylate cyclase activity in the pigeon erythrocyte lysate assay by Salmonella filtrates

Filtrate from:	cAMP (pmoles) stimulated in PEL assay experiment 1	experiment 2
uninoculated media	0.95	<0.8
10016	1.2	2.5
6158	4.52	2.2
986	2.76	1.8
9186	5.12	---
M206	---	4.5

D. Physical Relationship between *Salmonella* Endotoxin and Enterotoxin

Dorner (4) has reportedly dissociated a heat-labile enterotoxic factor from the endotoxin of a strain of *E. coli* which is enteropathogenic for humans. In the process of searching for an enterotoxigenic factor in culture filtrates of *Salmonella*, it was recognized that a large amount of endotoxin was elaborated by this organism. An attempt was made to dissociate heat-labile enterotoxin from *Salmonella* endotoxin. Through this type of experiment, we had thought the origin of heat-labile enterotoxin in *Salmonella* cells might be probed.

Purified endotoxin (Difco Laboratory) was treated with 1% SDS in 0.1M $(NH_4)HCO_3$, pH 8 for 4 hours at 37°C. Figure 3 shows the elution profile of the dissociated materials from an Agarose 5M column equilibrated with 0.1% SDS in 0.1M $(NH_4)HCO_3$, pH 8. The materials from all peaks were collected and SDS removed by column chromatography using ion-retardation resin AG 11 A8. The SDS-free materials were dialyzed in deionized water overnight and subsequently tested for the presence of enterotoxin with the CHO elongation assay (performed before the development of the CHO floating cell assay). As shown in Fig. 4, the percent elongation of CHO cells of the three dissociated materials was very low when compared to that of *Salmonella* 10016 culture filtrate. The background percent elongation of CHO cells exposed to deionized water was 18%. These data indicate that there is no physical relationship between endotoxin and enterotoxin.

E. *Salmonella* Cytotoxic Factor

During the initial stage of purifying the heat-labile enterotoxin, a factor was isolated from chromatography fractions of crude fermenter concentrates of *Salmonella* strain 9630. This factor appeared to be cytotoxic, causing rounding or cell death to CHO cells. The cytotoxic factor was further tested with Vero cells, a cell line commonly used for the detection of *E. coli* cytotoxin (20), and found to destroy the monolayered cells. Moreover, this factor could be partially destroyed by boiling for 30 minutes. Presently we do not know the significance of this factor, nor its relationship to the *Salmonella* toxin and whether or not it plays any role in the pathogenesis of salmonellosis. However, there is a possibility that this factor could be responsible in part for the invasive nature of *Salmonella*. We are presently in the process of developing a reliable and sensitive cytotoxin assay. This assay will make it possible to survey and select the cytotoxic strains of *Salmonella*. An electron microscopic study of the effect of this factor on cultured Henle intestinal epithelial cells as well as *in vivo* rabbit gut epithelial cells will be undertaken in the future.

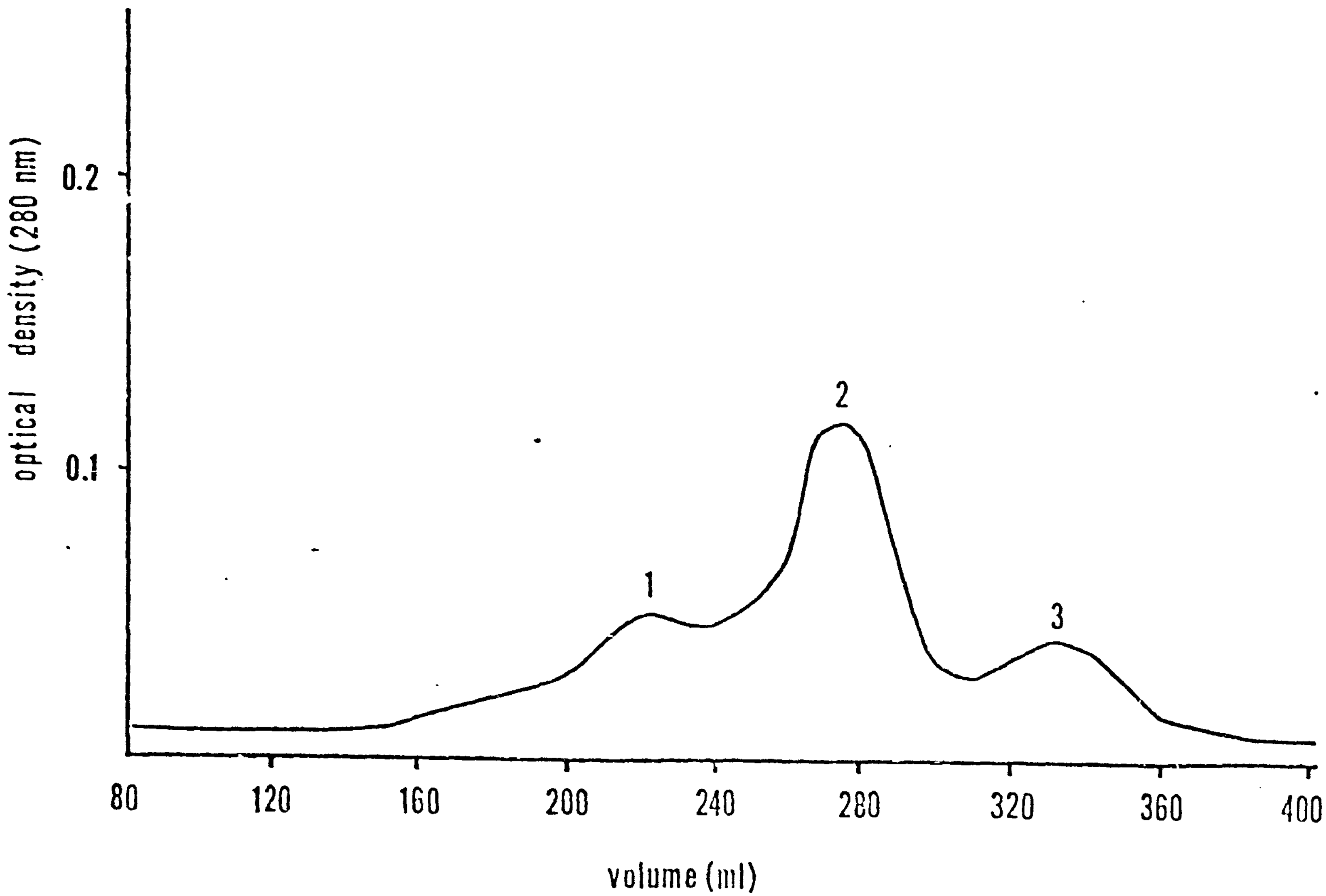


Fig. 3. Agarose 5M chromatography of purified Salmonella endotoxin. Endotoxin was treated with 1% SDS for 4 hours at 37°C, and eluted with 0.1% SDS in 0.1M $(NH_4)HCO_3$, pH 8.0. Numbers 1,2 and 3 indicate the dissociated materials. Enterotoxin activities were tested by CHO elongation assay.

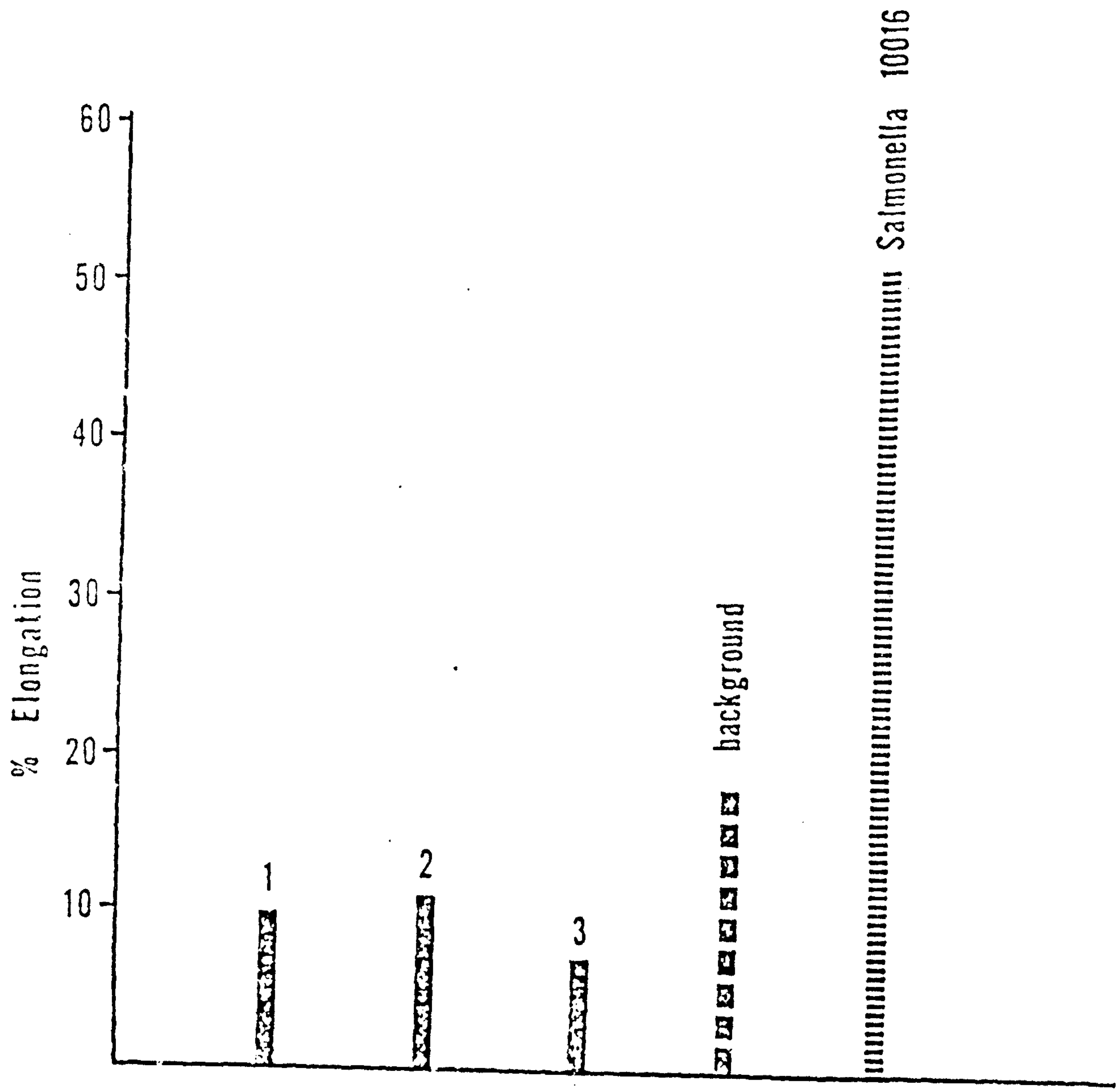


Fig. 4 . Enterotoxin activities of dissociated materials from *Salmonella* endotoxin as determined by CHO elongation assay. Dissociated materials (1,2,3) were dialyzed overnight in deionized water; background, deionized water; and *Salmonella* 10016, sterile 18-hr culture filtrate.

F. Plate Assays for Future Genetic Investigations

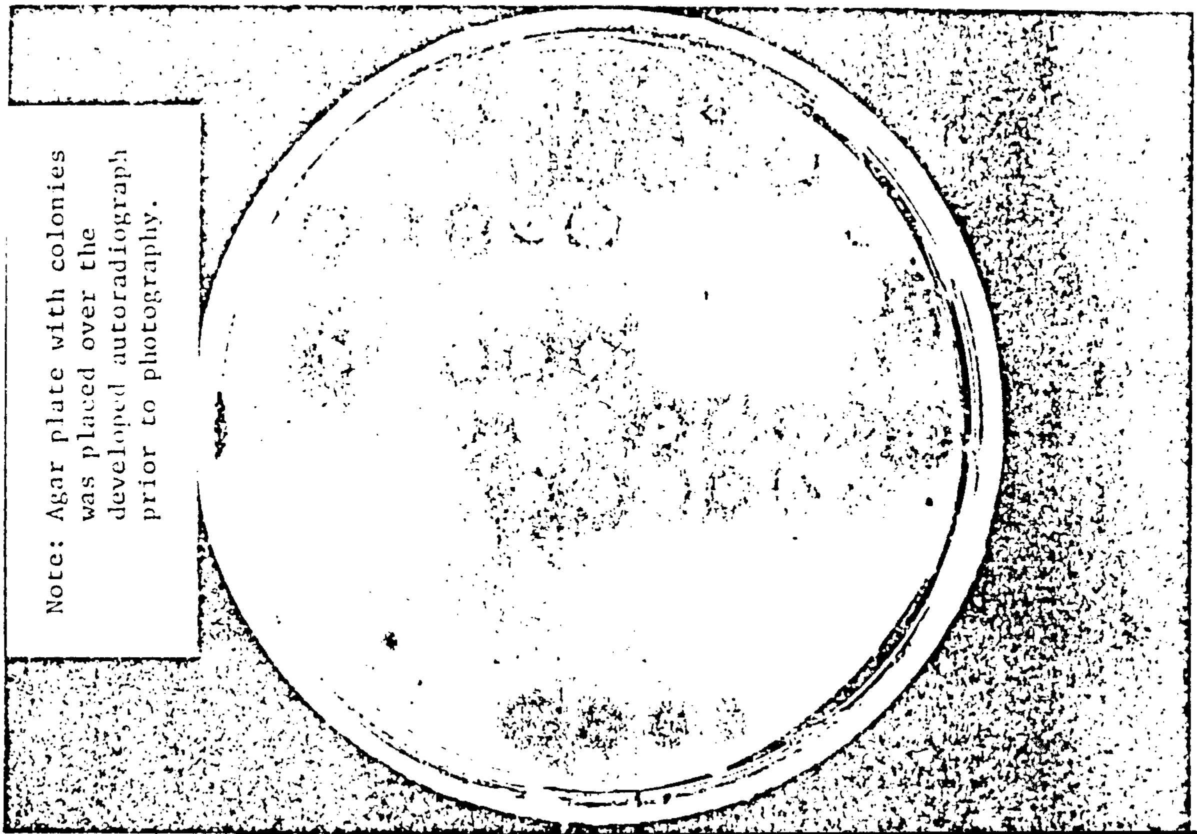
1. Blood Agar Plate Assay for Detection of Toxin Production by *Salmonella* Colonies

One major difficulty in studying the genetics of toxin production by *Salmonella* is the lack of a quick, inexpensive and relatively easy assay for testing many isolates for toxin production. For this purpose, we have recently developed a modification of the radial passive immune hemolysis assay of Bramucci and Holmes (1). Syncase agar plates are overlaid with 5 ml of molten syncase-blood agar containing 50-100 bacteria. The plates are then incubated at 37°C. After 24 hours, they are overlaid with 3 ml of molten syncase agar containing 100 μg of polymyxin B and 1 mg of lysozyme. The plates are then incubated for another 24 hours at 37°C. Treatment of the *Salmonella* colonies with polymyxin B and lysozyme causes them to release detectable levels of toxin. The toxin diffuses through the agar and binds to receptors on the surface of the erythrocytes in the agar. The plates are then overlaid with soft agar containing guinea pig complement and specifically purified antibody to cholera toxin. When the antitoxin and complement react with the toxin which has attached to the erythrocytes, the erythrocytes lyse, causing a zone of hemolysis around toxin-producing colonies, as shown in Figure 5. This may be an extremely valuable tool in screening for toxin production by mutants and recombinants derived in the course of genetic experiments.

2. Autoradiograph Plate Assay

Our laboratory has developed a second plate assay that uses cyanogen bromide (CNBr)-activated paper and specifically purified cholera antitoxin. Colonies of *Vibrio cholerae*, *Escherichia coli*, or *Salmonella* species are grown on CYE agar plates for 18-24 hours. The plates are inverted over a filter paper disc saturated with chloroform to enhance release of intracellular contents by lysis of the bacterial cells. Subsequently, a top agar layer, containing SDS and lysozyme, is applied to each plate. Plates are also spotted with 10 μl each of dilutions of cholera toxin to measure assay sensitivity. Whatman No. 1 filter paper discs, previously sensitized with specifically purified cholera antitoxin and stored at -20°C, are then laid on the surface of the agar overlay. After 9-10 hours of incubation, the sensitized paper discs are removed and washed in a Buchner funnel with PBS. Each is then floated in 10 ml of ^{125}I-labeled, specifically purified cholera antitoxin for 5 hours at room temperature. Following extensive washing again in PBS, the discs are dried and taped to x ray film for 1-2 days. The developed autoradiograph reveals the location of cholera toxin antigen surrounding colonies of toxinogenic strains (see Figure 6). Positive autoradiographic data is currently being obtained with strains of *Salmonella*, *Escherichia*, and *Vibrio*. The assay can detect as little as 1 ng of purified cholera toxin. We are currently assessing the feasibility of this assay for detection of toxin production from a variety of clinical isolates of *Salmonella* and *E. coli*, but will delay the presentation of our observations until more experience is acquired.

Figure 5



Note: Agar plate with colonies was placed over the developed autoradiograph prior to photography.

Figure 6

Like the other plate assay for toxin production, this assay appears to have considerable potential value.

G. Bacteriophage Investigations

Several strains of Salmonella, both toxin producers and nontoxin producers, were screened for the presence of temperate phages. The strains were subjected to mitomycin C induction (0.5 μ g/ml) while growing in CYE broth. Cell-free culture filtrates of the induced strains were dropped onto TSB agar plates containing bacterial indicator lawns of the same strains. Based on preliminary data, there did not appear to be a correlation between toxin production and phage association when MTC was used as the inducing agent. An experiment utilizing U.V. light as an inducer will be conducted to determine whether it is a more efficient phage inducer than MTC. A phage survey utilizing the U.V. induced phage preparations, along with a determination of toxin production for each strain, will be performed. Based on the results of this experiment, a more definitive statement may be made regarding any correlation between phage association and synthesis of heat-labile toxin in Salmonella strains.

A preliminary experiment was performed which involved inducing phage from a known lysogenic strain of Salmonella and constructing a new lysogen by infecting a non-toxin producing Salmonella recipient with the induced phage. Bacteriophage were U.V. induced from donor Salmonella strains W118-2, TML R66, and M206. Salmonella strains Thax-1 and 9SR2 were used as recipient lawns onto which drops of a cell-free phage preparation of each donor were placed. Portions of the area of lysis were harvested with a sterile wire loop and streaked onto quadrants of CYE agar plates. Two CYE agar plates, one containing a recipient strain of Salmonella in a soft agar overlay, were placed on grid templates. Twenty-four selected isolated colonies from the CYE agar streak plates were inoculated onto each of the two grid plates with sterile toothpicks. The plates were examined after 24 hours at 30°C for clearing around the colonies on the recipient lawn which indicated phage production, and, therefore, the formation of a new lysogen. The duplicate culture grown on the plain CYE agar plate was used as the donor for a repeat experiment. Six newly constructed lysogens were grown in CYE broth for 24 hours at 37°C and the filtrates and sonicates of each lysogen were assayed by the ELISA and the CHO floating cell assay. Salmonella toxin values for the newly constructed lysogens were lower than those of the original donor and recipient strains indicating perhaps that the bacteriophage associated with Salmonella strains utilized in this experiment did not provide the genetic information for toxin synthesis. Using a new phage inoculation device provided by Dr. P. Gemski, a larger number of Salmonella strains will be used as donors and recipients for the construction of new lysogens in the future and, hopefully, a definite conclusion regarding the possible role of bacteriophage in toxin production can be derived at that time.

H. Miscellaneous Observations

1. Suckling mouse assay - The suckling mouse assay (21) was set up in our laboratory to determine if crude filtrates containing toxin with CHO cell activity elicit a positive fluid response in this model. Although positive fluid responses were observed with filtrates from strains of E. coli known to elaborate ST, no fluid accumulation occurred in mice fed filtrates from several Salmonella strains, as illustrated in Table 7. We can conclude only that the Salmonella toxin is negative in this assay or that it was insufficient in concentration to elicit a positive response.

Table 7

Strain	Gut/Body Weight Ratio
E. coli C 22-1 filtrate	0.1280
Salmonella 732 filtrate	0.0564
Salmonella 6158 filtrate	0.0567
Salmonella 5931 filtrate	0.0580
Salmonella 6297 filtrate	0.0582
CYE Control Medium	0.0644

2. Adrenal Cell Assay - A collaborative arrangement was established with Dr. Sam Donta at the University of Iowa to measure toxin in 10 filtrate preparations. Salmonella culture filtrates were assayed by the CHO floating cell assay, as well as the ELISA prior to refrigerated shipment to Iowa. Dr. Donta assayed the 10 coded samples using the adrenal cell assay (22). He then assigned them a second code, added five additional samples, and returned them to us for reassay. We again assayed the samples as a blind study and compared these results with the initial titrations. The data from this study is summarized in Table 8. It should be pointed out that no positive data with Salmonella filtrates was obtained by Dr. Donta using the adrenal cell assay. In contrast, the CHO cell assay and ELISA correlated well in estimates of Salmonella toxin content. Furthermore, the blind assay correlated quite well with the initial titrations. We cannot explain the lack of positive results in the adrenal cell assay. No further studies are planned, but the lack of adrenal cell responses may reflect a difference in Salmonella and cholera toxin or it may simply depict a difference in assay sensitivity.

3. Mouse LD_{50} Determinations - A study was performed to determine if a correlation existed between the capacity of Salmonella strains to produce the heat labile toxin and lethal infection of mice. Adult Swiss Webster mice were distributed into groups of 8 mice. Doses of viable cells of each Salmonella strain, ranging from 10^0-10^5, were injected by the intraperitoneal route without addition of mucin. The mice were examined daily and deaths were recorded for 21 days following injection. The LD_{50} values observed are as follows in Table 9:

Table 8
Titration of *Salmonella* Culture Filtrates

Sample	CHO Floating Assay C.T. Equivalents ng/ml Initial Titration	CHO Floating Assay C.T. Equivalents ng/ml Coded Titration	ELISA C.T. Equivalents ng/ml Initial Titration	ELISA C.T. Equivalents ng/ml Coded Titration	Adrenal Cell Assay
Sal. 8994	8.9	6.5	5.76	6.5	-
Sal. 8994MTC	29.0	5.1	59.0	39.0	-
Sal. 10016	4.1	5.7	12.5	3.0	-(questionabl
Sal. 10016MTC	50.8	31.1	26.5	50.3	-
Sal. SL1027	53.9	26.3	18.5	20.5	-
Sal. SL1027MTC	111.2	31.1	46.0	50.3	-
Sal. SR11	6.5	11.3	13.5	0	-
Sal. SR11MTC	10.1	27.0	67.0	36	-(Graininess)
CYE Medium	<0.2	13.5	0.36	2.8	-
CYE Medium+MTC	0.7 (retest <0.2)	8.3	0.36	0	-(Graininess)
BHI Medium	N.T.	2.7	N.T.	2.6	-
V. cholerae (Rita Caldwell strain) (tox-) BHI	N.T.	0.3	N.T.	2.7	-
V. cholerae 569B (BHI)	N.T.	30.2	N.T.	2.1	+
Salmonella sp. Group B BHI	N.T.	5.3	N.T.	2.8	-
Salmonella sp. Group D1 BHI	N.T.	4.6	N.T.	2.5	-

N.T.=Not Tested

Table 9

Salmonella isolate	LD_{50}	Toxin Producing CHO Cell Activity**
RIA	$>10^5$	33.4
9186	$>10^5$	3.6
9630	$>10^5$	20.7
10016	$>10^5$	65.0
10234	$>10^5$	N.D.
2000	10^4	61.4
986	652	N.D.
SR11	163	26.7

* No deaths were recorded in any of these *Salmonella* groups even at 10^5 cells/ml.

** Cholera toxin equivalents (ng/ml) in cell sonicates from a CYE broth culture (from Peterson, J.W., C.W. Houston, and F.C.W. Koo. 1980. Factors affecting synthesis and release of *Salmonella* toxin. Submitted to J. Bacteriol.)

Although the above data suggest that the *Salmonella* toxin plays little role in systemic infection leading to animal death, an additional experiment was designed to examine its possible involvement further. Adult, Swiss Webster mice were distributed into three groups: Nonimmunized controls, immunized 1 μg cholera toxin, or immunized 10 μg cholera toxin. The cholera toxin immunized mice received the toxin dose in 0.5 ml of Tris buffer by the intraperitoneal route two weeks prior to live cell challenge. All mice were challenged with varying doses of live *Salmonella* SR 11 by the intraperitoneal route. The following data were derived (Table 10):

Table 10

Challenge Group	LD_{50}*
Immunized cholera toxin (10 μg)	1000
Immunized cholera toxin (1 μg)	100
Nonimmunized control	588

*viable *Salmonella* SR11 injected IP

The data indicate that cholera toxin did not protect mice against live cell *Salmonella* challenge. Based on these data we elected not to pursue further studies relating the *Salmonella* toxin to lethal infection.

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Annual Progress Repc - Additional Progress Section

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